THE RETENTION OF NITROGEN, CALCIUM, PHOSPHORUS, AND MAGNESIUM BY PREGNANT WOMEN.*

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HISTORICAL.

Two methods of investigation throw light upon the storage of different elements by pregnant women—analyses of fetuses which have been obtained because of miscarriage or other accident, and balance experiments carried out on the women themselves.

Fetal Analyses.—As early as 1877 Fehling (1) reported the gross chemical composition of eleven human fetuses. Since then Hugounenq (2) has published analyses for the mineral constituents of eight fetuses; Michel (3) the calcium, phosphorus, magnesium, and nitrogen content of seven; Schmitz (4) the ash and calcium content of twenty; and Givens and Macy (5) the calcium and magnesium of twenty-five. Camerer (6) gave the carbon, hydrogen, nitrogen, and complete ash analyses of six full term infants.

Although the results from different workers vary considerably, they do furnish indication of the probable demands of the human fetus at different stages of development. Some of the data have, therefore, been used by the authors of this paper to calculate average daily increments in mineral deposits at different periods of fetal development, and the resulting curves have been included on

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the charts for comparison with the actual retentions by the subjects in the present investigation.

Balance Experiments.—The literature records only three important balance experiments on pregnant women and few minor ones giving figures only on the nitrogen retention during the last few days before delivery. The latter are not summarized here.

Hoffström's (7) is the most extensive set of observations on a single individual. He studied the retention of all minerals and nitrogen by one woman over the entire last 23 weeks of pregnancy. His subject had rather high nitrogen intakes ranging from 10 to 17 gm. per day and an average retention of 1.84 gm. per day during the last 10 weeks. Her calcium intake, relatively even higher than the nitrogen in comparison with American dietary habits, was often over 2.0 gm. per day, and averaged 1.7 gm.; the retention averaged 0.08 gm. during the third quarter of pregnancy, and 0.32 gm. during the last 10 weeks. With the phosphorus intake averaging 2.0 gm. per day, the retention was 0.30 gm. for the last 20 weeks.

Landsberg (8) observed the nitrogen, phosphorus, and sulfur retentions by six women at intervals during the last half of pregnancy. His subjects had nitrogen intakes from 12 to 18 gm. daily, and retentions from 1.4 to 3.9 gm., the retention paralleling the intake. The phosphorus intake was around 1.8 gm. per day, the retention 0.20 to 0.36 gm., only eight balances being reported on the six women.

Wilson (9) made continuous observation of the nitrogen metabolism in two women during the last 22 and 16 weeks of pregnancy, respectively. The nitrogen of the food, which he merely computed from published figures, ranged from 15 to 19 gm. per day, and the retention from 1.1 to 6.7 gm. The study tends to support the evidence from Landsberg that the higher retentions are coincident with higher intakes.

Thus observations on calcium retention are reported for one woman only (Hoffström's extensive series); on phosphorus retention for seven women, including Hoffström's; and on nitrogen for the same seven and two others. To be added to these are the numerous observations on the calcium and phosphorus retentions of three women in the paper by Hunscher and Macy[1] in this number of the *Journal.*

EXPERIMENTAL.

Subjects.—In the present investigation an attempt has been made to study several pregnant women under their own chosen

[1]Macy, I. G., Hunscher, H. A., Nimms, B., and McCosh, S. S., *J. Biol. Chem.*, **86,** 17 (1930).

régime of dietary habits and other living conditions. A total of twenty-three 4 to 6 day metabolism studies has been carried out on nine different women from the 11th to the 39th weeks of pregnancy. On two of the women five balance periods each were observed, on three others three periods each, and on four women one period only. Different social and economic levels were represented, the homes having incomes ranging from 25 dollars a week up. Three of the women were high school graduates, six were college graduates. The genuine interest of the women in knowing what was actually going on in their cases as well as in the whole study and the regularity of the collections from day to day and from period to period were some of the indications of complete cooperation on the part of the subjects. Further information about the subjects and points in regard to their babies are given in Table VI.

Methods.—The food to be eaten was weighed and sampled by the investigator in the home at meal time, and later the samples pooled into a composite representing one-tenth of the intake for the period. The total feces for the period were collected and dried, carmine markers being used to make separations. Aliquots of the daily urine collections were made into composites and preserved with toluene and acid.

Analyses for total nitrogen were carried out by the Arnold-Gunning modification of the macro-Kjeldahl procedure. Calcium and magnesium were determined gravimetrically according to the McCrudden (10) method with control of pH as recommended by Shohl (11), and the double precipitation for magnesium as described by Epperson (12); phosphorus was determined according to the Neumann (13) method of moist oxidation, recently emphasized by Lundell and Hoffman (14), and the double precipitation procedure of McCandless and Burton.

RESULTS AND DISCUSSION.

Nitrogen.—The nitrogen intake, output, and retention of the nine women are presented in Table I, and the retention shown graphically in Chart I.

Probably the first conclusion to be drawn from a glance at Chart I is that the storage is markedly irregular, both from woman to woman and for the same woman at different times. The amounts

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retained varied from −0.43 to +8.45 gm. per day, some women storing 10 to 15 times as much as others. Neither did the storage seem related to any definite period of pregnancy, but reached a

TABLE I.

Nitrogen Balances in Pregnant Women (Daily Averages).

Case and period No.	Wk. of pregnancy.	N intake.	N output.			Balance.
			Urine.	Feces.	Total.	
		gm.	gm.	gm.	gm.	gm.
A 1	12	8.16	5.46	1.84	7.30	+0.86
2	20	13.97	9.04	2.21	11.25	+2.72
3	28	13.56	5.84	2.62	8.46	+5.10
4	33	10.04	6.67	2.07	8.74	+1.30
5	39	8.13	5.10	1.67	6.77	+1.36
B 1	16	10.41	6.97	1.77	8.74	+1.67
2	22	11.54	7.40	1.88	9.28	+2.26
3	29	10.91	7.07	1.61	8.68	+2.23
4	35	9.84	7.18	1.75	8.93	+0.91
5	39	10.03	7.97	1.64	9.61	+0.42
C 1	27	10.79	8.23	1.80	10.03	+0.76
2	33	12.58	7.16	1.76	8.92	+3.66
3	37	13.19	8.16	1.62	9.78	+3.41
D 1	30	10.63	7.34	1.72	9.06	+1.57
2	34	9.12	7.12	1.64	8.76	+0.36
3	38	9.57	7.16	2.84	10.00	−0.43
E 1	11	13.80	6.91	1.79	8.70	+5.10
2	21	14.31	4.18	1.68	5.86	+8.45
3	32	12.91	9.27	1.85	11.12	+1.79
F	15	10.65	8.28	2.21	10.49	+0.16
G	29	10.70	6.46	1.70	8.16	+2.54
H	36	7.74	5.09	1.67	6.76	+0.98
I	39	11.21	7.21	1.35	8.57	+2.64

high peak in the early months for some women and in the last months for another.

In most cases, however, there was a tendency to high nitrogen retention in the middle of pregnancy, followed by a lowering in the

later months. This may have been due to one or more of three possible factors: an undernourished condition in the mother previous to or at the beginning of pregnancy; the growth of the placenta and hypertrophy of other maternal organs; or often to a deliberate restriction of protein intake in the later months upon the advice of the physician (Cases A, D, E, and H).

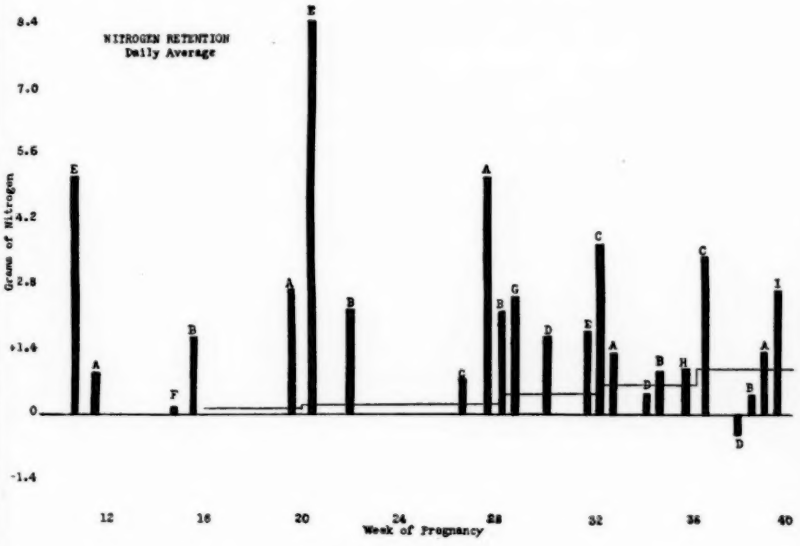

CHART I. Daily nitrogen retention by nine pregnant women at different weeks of pregnancy. The horizontal line represents the average daily increment to the fetus as computed from published fetal analyses.

Indeed, the most frequently observed relation, and one which helps to explain most of the irregularities in storage, is that between nitrogen intake and retention. The highest retentions in the group as a whole accompanied the highest intakes, and individual women stored more when ingesting more. Case E most consistently illustrates this relationship, her very high retention coming when her intake was highest and her comparatively low retention when the intake was lowest; but every woman on whom there

was more than one observation furnishes one or more instances of similar effect of varying the nitrogen of the diet (Case A, Periods 1 and 2; Case B, Periods 3 and 4; Case C, Periods 1 and 2; Case D, Periods 1 and 2). For the only case (Case C) where the food nitrogen remained fairly high until the end of pregnancy, the retention remained high also.

Two other less general conclusions may be drawn from the nitrogen figures. The first has already been mentioned: the effect of undernutrition, seen particularly in Cases E and A. Case E who before her pregnancy had been anemic and below weight gained 35 pounds during her first 8 months, and Case A had suffered from severe nausea just before her first balance observations, so that the very high retentions reported for these two are what might be expected.

The second point is that the subject with the most consistently low nitrogen balance and the only negative balance of the whole group (Case D) was eating a large amount of *raw* vegetables and fruits (carrots, cabbage, lettuce, celery, apples, etc.). In the last period of her three when she was failing to store any one of the elements studied, she had mild diarrhea with three stools daily instead of the single stool of her early periods, and a coefficient of digestibility for nitrogen of 70 instead of her earlier 82.

Comparison of the figures of all of these women with those in the literature shows that intake and storage are both from 30 to 40 per cent lower than published figures, but that the main conclusion from the authors' observations, that high storage usually follows high intakes and low storage low intakes, is in accord with the results of Landsberg and of Wilson.

Calcium.—Retentions of calcium are given in Table II, and are represented graphically in Chart II. The supposed fetal demand is indicated on Chart II by two lines, the lower one calculated from Schmitz's figures for fetal calcium, and the upper dotted line from Hugounenq's.

Two facts are at once evident from Chart II. First, calcium retention, in contrast to that for nitrogen, is highest at the end of pregnancy rather than near the middle, and is more consistently related to the period of gestation. A curve which may be taken as a tentative standard for calcium retention, based on eleven observations of the three subjects storing the larger quantities

(Cases A, B, and I) begins to rise about the 4th month, soon reaches 0.10 gm. per day, continues gradually to about 0.20 gm. by the 9th month, then more steeply to 0.30 gm. by the end of the

TABLE II.

Calcium Balances in Pregnant Women (Daily Averages).

Case and period No.	Wk. of pregnancy.	Ca intake.	Ca output.			Balance.
			Urine.	Feces.	Total.	
		gm.	gm.	gm.	gm.	gm.
A 1	12	0.727	0.201	0.584	0.785	−0.058
2	20	1 624	0.377	1.103	1.480	+0.144
3	28	1.491	0.263	1.116	1.379	+0.112
4	33	1.314	0.267	0.900	1.167	+0.147
5	39	1.192	0.156	0.750	0.906	+0.286
B 1	16	1.096	0.417	0.603	1.020	+0.076
2	22	1.214	0.326	0.767	1.093	+0.121
3	29	0.784	0.313	0.286	0.599	+0.185
4	35	1.026	0.324	0.498	0.822	+0.204
5	39	0.745	0.191	0.316	0.507	+0.238
C 1	27	0.716	0.103	0.585	0.688	+0.028
2	33	1.078	0.103	0.891	0.994	+0.084
3	37	1.346	0.120	1.058	1.178	+0.168
D 1	30	1.125	0.121	0.961	1.082	+0.043
2	34	1.182	0.150	0.961	1.111	+0.071
3	38	1.044	0.122	0.953	1.075	−0.031
E 1	11	1.152	0.275	1.015	1.290	−0.138
2	21	1.705	0.245	1.317	1.562	+0.143
3	32	1.508	0.272	1.198	1.470	+0.038
F	15	0.790	0.473	0.431	0.904	−0.114
G	29	0.603	0.085	0.477	0.562	+0.041
H	36	0.694	0.125	0.458	0.583	+0.111
I	39	1.046	0.313	0.427	0.740	+0.306

10th month. These figures are similar to those of Hoffström's subject in the middle months of pregnancy, but his subject in the last 10 weeks actually averaged 0.32 gm. per day, whereas no individual woman in the present study reached this figure at any time.

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The second conclusion from Chart II is that the women were not storing in excess of fetal demands. The tentative standard curve formulated above from the three women with highest storage corresponds almost exactly with that for fetal requirement, but all except one of the other figures fall below—most of them far below. The indications, therefore, are that most of these women either

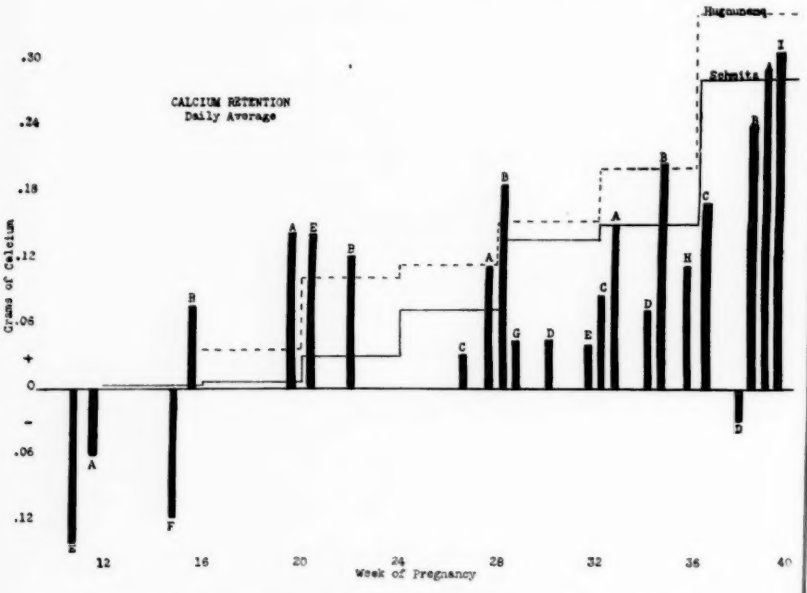

CHART II. Daily calcium retention by nine pregnant women at different weeks of pregnancy. The horizontal lines represent the average daily increments to the fetus as computed from published fetal analyses.

were depositing only small amounts of calcium in the fetus or were using their own reserves to meet fetal needs.

There seem to be several possible explanations of the low storage, chiefly dietary. In every case, save one, where the daily intake of calcium was below 1 gm., the storage of calcium was also low. Other explanations are associated with the character of the diet as summarized in Table III, especially that of the woman with

consistently low storage and the only negative balance toward the end—Case D. She had excessively high crude fiber as already stated and probably very low vitamin D, since no provision was made for this factor in the diet and she passed the last months of pregnancy in midwinter in Chicago.

The possible factor of the acid-base reaction of the diet is also suggested, because Case C regularly excreted the most acid urine, pH as low as 4.8, and Case D the most alkaline, pH as high

TABLE III.

Summary of Dietary Characteristics of Experimental Women.

Case.	Milk daily.	Fresh fruits and vegetables daily.	Average No. eggs per wk.	Meat and fish, average No. servings per wk.	Remarks.
A	1 qt.	++++	3	1	
B	1 pt.	+	1	2	No milk in Periods 3 and 5.
C	1 "	+	8–10	7–12	Very little milk in Period 1.
D	1 "	++++	1	1	Fruits and most vegetables raw. No meat the last month.
E	1 qt.	+++	4	3	Highest total intake.
G	1 pt. (wk.)	++	3	7	Smallest " "
H	1 qt. "	+++	3	2	Small servings of meat.
I	1 pt.	+++	6	7	

as 7.2, of any of the subjects studied, and there was reason to believe that the diets were as far from neutrality as the urine indicated.

Cod liver oil was taken by only two of the women, Case A daily for 9 weeks including Periods 3 and 4, and Case E for 3 weeks previous to and during Period 3 but only half a teaspoonful three times a week. No marked effects were noticed, but Case A's good storage was maintained although her intake went down somewhat. The results, therefore, prove little as to the value of cod liver oil in pregnancy, but do invite further investigation.

Phosphorus.—Table IV and Chart III giving data of phosphorus retention show clearly the tendency to high storage in early middle pregnancy, followed by a fairly constant level during the remainder

of gestation. Case B illustrates this with an almost constant level of storage for the 23 weeks observed. Among the variations from this tentative straight line standard is Case C who exhibits a sharp

TABLE IV.

Phosphorus Balances in Pregnant Women (Daily Averages).

Case and period No.	Wk. of pregnancy.	P intake.	P output.			Balance.	Ca : P ratio.	
			Urine.	Feces	Total.		Intake.	Stored.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*		
A 1	12	0.948	0.652	0.436	1.088	−0.140	0.77	
2	20	2.065	1.011	0.742	1.753	+0.312	0.78	0.46
3	28	1.868	0.642*	0.697	1.339	+0.529	0.80	0.21
4	33	1.552	0.778	0.560	1.338	+0.214	0.85	0.68
5	39	1.288	0.568	0.509	1.077	+0.211	0.92	1.35
B 1	16	1.328	0.694	0.422	1.116	+0.212	0.82	0.36
2	22	1.618	0.855	0.574	1.429	+0.189	0.75	0.64
3	29	1.139	0.650	0.271	0.921	+0.218	0.69	0.85
4	35	1.210	0.612	0.408	1.020	+0.190	0.85	1.07
5	39	1.227	0.649	0.346	0.995	+0.232	0.61	1.03
C 1	27	1.147	0.741	0.323	1.064	+0.083	0.62	0.34
2	33	1.492	0.715	0.562	1.277	+0.215	0.72	0.40
3	37	1.687	0.830	0.566	1.396	+0.291	0.80	0.57
D 1	30	1.375	0.712	0.681	1.393	−0.018	0.82	
2	34	1.329	0.628	0.679	1.307	+0.022	0.89	0.32
3	38	1.248	0.650	0.686	1.336	−0.088	0.84	
E 1	11	1.562	0.835	0.776	1.611	−0.069	0.74	
2	21	2.210	0.925	0.928	1.853	+0.357	0.77	0.40
3	32	1.858	0.927	0.815	1.722	+0.116	0.81	0.30
F	15	1.287	0.731	0.501	1.232	+0.055	0.61	
G	29	0.942	0.432	0.337	0.769	+0.173	0.64	0.24
H	36	1.193	0.521	0.518	1.039	+0.154	0.58	0.72
I	39	1.336	0.744	0.360	1.104	+0.232	0.78	1.30

* This figure is uncertain.

retention rise in the last 13 weeks, but this is coincident with her high nitrogen storage which continued to the end of gestation.

The phosphorus balances on these cases compare favorably with

those in the literature. The amounts retained by the best cases (Cases A, B, C, and I) are only a little below the 0.2 to 0.3 gm. daily retained by the subjects of earlier workers, and the plateau of retention of Cases B and A is shown also by Hoffström's subject. The phosphorus intakes of the women in this paper, however, are more comparable with those of earlier subjects than was true for calcium.

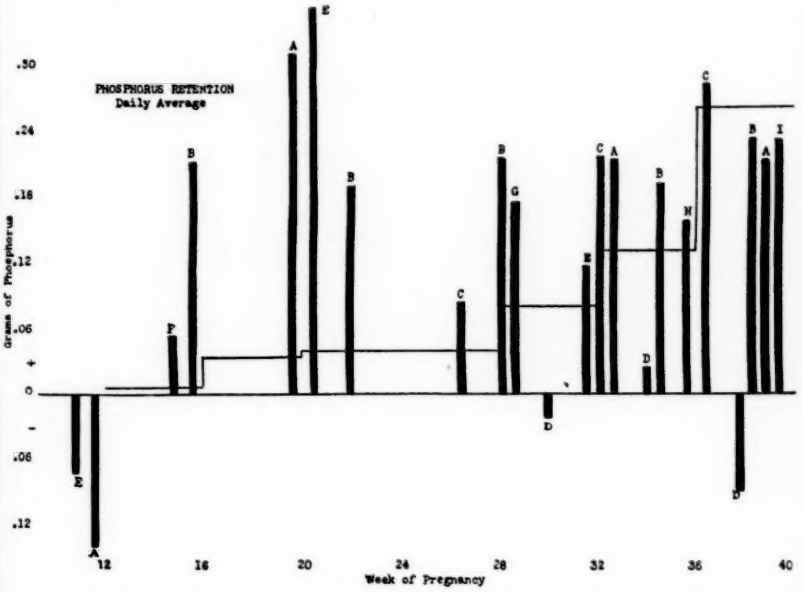

CHART III. Daily phosphorus retention by nine pregnant women at different weeks of pregnancy. The horizontal line represents the average daily increment to the fetus as computed from published fetal analyses.

Calcium-Phosphorus-Nitrogen Relationship.—Charts I to III suggest definitely that the high phosphorus retention in early pregnancy is related to the high nitrogen storage of the same period, but that in later pregnancy, when the nitrogen rate has fallen off, the phosphorus retention continues high, apparently associated with the greater calcium deposition of this period.

The ratio of calcium to phosphorus in the intake was fairly

constant, ranging in most cases from 0.8 to 0.9 (Table IV) and never above 1.0. Lower ratios, from 0.6 to 0.7, were usually associated with low intake of milk. The ratio of the amounts retained seemed in no way related to the intake, but ranged from

TABLE V.

Magnesium Balances in Pregnant Women (Daily Averages).

Case and period No.	Wk. of pregnancy.	Mg intake.	Mg output.			Balance.	Ca:Mg ratio intake.
			Urine.	Feces.	Total.		
		gm.	gm.	gm.	gm.	gm.	
A 1	12	0.236	0.092	0.158	0.250	−0.014	3.1
2	20	0.470	0.153	0.236	0.389	+0.081	3.4
3	28	0.478	0.152	0.284	0.436	+0.042	3.1
4	33	0.657	0.179	0.505	0.684	−0.027	2.0
5	39	0.810	0.208	0.913	1.121	−0.311	1.5
B 1	16	0.275	0.107	0.155	0.262	+0.013	4.0
2	22	0.339	0.114	0.214	0.328	+0.011	3.5
3	29	0.278	0.092	0.126	0.218	+0.060	2.8
4	35	0.294	0.119	0.169	0.288	+0.006	3.5
5	39	0.466	0.141	0.328	0.469	−0.003	1.6
C 1	27	0.252	0.154	0.115	0.269	−0.017	2.8
2	33	0.243	0.139	0.130	0.269	−0.026	4.4
3	37	0.282	0.155	0.110	0.265	+0.017	4.7
D 1	30	0.318	0.147	0.244	0.391	−0.073	3.5
2	34	0.371	0.100	0.244	0.344	+0.027	3.2
3	38	0.409	0.148	0.309	0.457	−0.048	2.5
E 1	11	0.358	0.092	0.292	0.384	−0.026	3.2
2	21	0.480	0.094	0.350	0.444	+0.036	3.5
3	32	0.411	0.096	0.318	0.414	−0.003	3.6
F	15	0.280	0.124	0.171	0.295	−0.015	2.8
G	29	0.269	0.090	0.166	0.256	+0.013	2.2
H	36	0.321	0.116	0.206	0.322	−0.001	2.1
I	39	0.328	0.090	0.175	0.265	+0.063	3.2

about 0.4 in the early months to 1.3 in the last month (Cases A, B, and I). Many of the women, however, did not reach this maximum ratio because calcium retention remained low. The 1.3 compares favorably with the retention ratio observed in infants

under 1 year of age (15) but is somewhat below the 1.6 ratio for the calcium-phosphorus content of the mature human fetus as shown by Camerer's analyses. This discrepancy is additional evidence that calcium retentions were too low, and also that phosphorus may be diverted to uses other than the fetus, even in late pregnancy.

Magnesium.—One of the chief aims in following the magnesium balances in this series of investigations was a determination of the possible effect of excess magnesium ingestion upon calcium retention. Table V gives a column showing the calcium-magnesium ratio in the intakes, thus indicating when the proportions of magnesium were high.

The average daily magnesium intake varied from 0.24 to 0.81 gm. per day, but over half of the number were between 0.27 and 0.37 gm., the ratio of calcium to magnesium thus being usually 3.0 to 4.0. Lower ratios of 2.0 to 1.5 resulted from the ingestion of magnesium in Bisodol or milk of magnesia, for medicinal purposes (Case A, Periods 4 and 5; Case B, Period 5) but similar ratios occurred when the calcium of the diet was exceptionally low (Cases G and H). The calcium retention curves of Cases A and B show no indication of untoward effects from the higher magnesium intakes.

The retention of magnesium itself was irregular and inconsistent. There was a tendency to more and larger negative balances in the last of pregnancy.

Roentgenograms of Babies.—Roentgenograms of the wrists and ankles of most babies were obtained on the 8th day after birth, with the idea of observing differences dependent upon prenatal life before exposure to postnatal influences. Clear, though not marked, distinctions could be seen and were closely paralleled by the mother's good or bad retention, especially of calcium. The poorest cases of bone formation came from pregnancies in which both calcium and phosphorus retentions were low (Cases D and H). In these the distal end of the ulna showed a tendency toward cupping and the ends of the other long bones toward flattening; the epiphyseal ends of the diaphyses were poorly calcified. The best development was in Baby C, whose bones were well calcified and the ends normally rounded or pointed. (It must be remembered that no very late balance was obtained on Case C.) Babies

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TABLE VI.

Summary of Protocols.

Case.	Mother.				Baby.			
	Description.	Comment.	Tooth destruction.	Lactation first 3 mos.	Date of birth.	Birth weight.		Roentgenograms at 8 days.
						lbs.	*oz.*	
A	I para, 22 yrs., 60 in., 125 lbs. Gain during pregnancy 35 lbs.	Nausea 4th–12th wks., cod liver oil 25th–34th wks., $NaHCO_3$ Periods 3, 4, Bisodol Periods 4, 5, $Mg(OH)_2$ Period 5.		+++	6- 8-28	7	12	Fair.*
B	II para, 27 yrs., 64 in., 150 lbs. Gain 20 lbs.	Gastric disturbance last month.	One abscess 10th wk. No losses.	++	4- 2-29	7	4	Fair.
C	II para, 24 yrs., 66 in., 128 lbs.	Influenza 31st wk.	One sensitive to heat; one chipped off.	++++	2-27-29	6	13	Good.
D	I para, 26 yrs., 64 in., 140 lbs. Gain 35 lbs.	Intestinal disturbance last period.	Two chipped off.	++	2-26-29	6	15	Poor.
E	I para, 29 yrs., 66 in., 130 lbs. Gain 40 lbs.	Previously anemic, below weight, cod liver oil 30th–36th wks.		++++	6- 6-29	8	3	
G	IV para, 35 yrs., 60 in., 117 lbs. Gain 28 lbs.			++++	7- 7-28	8	13	Fair.
H	I para, 28 yrs., 65 in., 114 lbs. Gain 28 lbs.	Below weight.		++	10- 2-28	6	15	Poor.
I	II para, 32 yrs., 60 in., 122 lbs. Gain 23 lbs.		Has no teeth.	++++	11-13-27	7	0	

*See descriptions of roentgenograms of babies.

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A, B, and G ranged between these extremes, and so did their mothers with respect to phosphorus retention, and Case A and B ranked highest in calcium retention.

Lactation.—High nitrogen retention, particularly in the last half of pregnancy, was followed by success in lactation (Cases C and I) and low retention usually preceded partial failure in lactation (Cases B, D, and H, Table VI). Thus it seems from these few cases that restricted protein intake does not permit the construction of a protein reserve in preparation for milk flow, may even deplete any reserves already present, and thereby preclude successful performance.

SUMMARY.

A series of twenty-three balance experiments on nine women at different stages between the 11th and 39th weeks of pregnancy showed considerable irregularity of behavior, both from woman to woman and for the same woman at different periods.

The nitrogen retention tended to vary chiefly with the intake. It was often higher in midpregnancy than later, due, in different cases, to voluntary restriction of protein intake in later months, or to an undernourished condition of the mother at the beginning of pregnancy. Both intake and retention of nitrogen by these women were 30 to 40 per cent lower than those of women observed a quarter of a century ago.

The calcium retention was also somewhat irregular but showed marked tendency to rise toward the end of pregnancy. A tentative standard curve, based on eleven observations of the three subjects storing the most, begins to rise about the 4th month, continues gradually to about 0.20 gm. daily by the 9th month, then somewhat more steeply to 0.30 gm. by the last week of pregnancy. The amount stored by most of the women was far below this curve and below a similar one computed from published analyses of fetuses, as well as much below the only other case recorded in the literature.

Phosphorus retention tended to rise to 0.15 to 0.20 gm. per day by the 5th month and then remained at a level of 0.20 to 0.30 gm. until the end of pregnancy. It corresponded to periods of high nitrogen retention in midpregnancy and to periods of high calcium retention toward the end.

*See descriptions of roentgenograms of babies.

XUM

High magnesium intake, sufficient to change the calcium-magnesium ratio from 3.5 to 1.5, seemed to have no effect on calcium retention.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE.

I. CALCIUM AND PHOSPHORUS UTILIZATION IN PREGNANCY.

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As the studies on the factors influencing the secretion of human milk have progressed since 1924 it has become increasingly apparent that there are many variables operating in the metabolism of women during the child-bearing and child-rearing stages. This paper is the initial report of a series of investigations on the metabolism of women during the entire reproductive cycle, including both pregnancy and lactation. In this preliminary report, data concerning the calcium and phosphorus metabolism of three women at different stages of the gestation period are given with the primary interest of observing the metabolic response of the maternal organism to the physiological changes coincident with the development of the fetus and the preparation for lactation.

Animal experiments and clinical observations indicate that notable metabolic demands are placed upon the maternal organism during the reproductive cycle. It is important to know whether certain losses manifest themselves in the mother herself or in her offspring. Furthermore, it is essential to learn whether specific care, the right kind and amount of exercise, good hygiene, a satisfactory state of mind, and an adequate dietary can reduce the untoward effects that may be revealed in the mother

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and reflected in the child during the reproductive cycle. In other words, can the mother be given a preparation for child-bearing and child-rearing which will benefit both her and the child?

The requirements for reproduction are many. The literature dealing with fetal mineral requirement in relation to the metabolism of women in pregnancy has been adequately reviewed and discussed by Hoffström (1), Murlin (2), Shohl (3), Harding (4), Lusk (5) and, more recently, by Coons and Blunt (6). Since the fetal demands have been reviewed, it is our purpose to consider metabolism during gestation in the light of other factors that may have similar suppletory relations to the utilization of calcium and phosphorus.

The pregnant woman undergoes a marked physiological adaptation when the condition of rapid growth is suddenly superimposed upon her. Unquestionably, many metabolic changes are set up within her body to take care, simultaneously, of the unaccustomed nutritional demands of the fetus and the preparation of her own tissues for future elaboration of milk. Orr and Magee (7) have recently stated that "the products of conception can, apparently, of themselves, induce retention of inorganic elements but there is no evidence to show whether the retention due to this stimulus is sufficient to meet the demands of the uterine contents alone or lactation as well."

There are at least two methods of studying the requirements of the pregnant woman. One is to determine the metabolic balances at definite periods; the other, to study the amount of materials actually laid down in the human fetus and its adnexa at similar intervals. The calcium and phosphorus balances of a woman from the 17th week throughout pregnancy have been recorded by Hoffström (1), one case by Landsberg (8), and one from our own laboratory (9). Coincident with the present paper, Coons and Blunt (6) are reporting twenty-three balances, ranging from the 12th week to the 9th month in pregnancy, on nine different women. Calcium and phosphorus analyses of fetuses of various ages have been recorded and reviewed by Michel (10), Givens and Macy (11), Schmitz (12), Shohl (3), and others. In view of the above accounts it would be untimely and unnecessary to include a discussion of these very important phases. Moreover, Murlin (2), Marshall (13), and Harding (4) in their excellent expositions of

the metabolism of the mother and offspring have given comprehensive discussions of many of the factors entering into maternal metabolism. It has been our aim to observe and record some of the physiological changes occurring simultaneously during the development of the human fetus and the maternal preparation for lactation, and to indicate other possible factors operating at the time of these periods of acquisition.

During the reproductive cycle many compensatory physiological changes, other than those of the utilization of materials actually laid down in the fetus itself, take place. The total retention of elements usually exceeds the requirements of the fetus and a large proportion of mothers find themselves more physically fit at the end of pregnancy than at the beginning. A maternal physiological readjustment is stimulated by the products of conception. Certain portions and organs of the body are temporarily quickened into functional activity in anticipation of the claims made by the growing fetus and its adnexa together with the specific demands for the periods of labor and lactation. The significance of this general purposeful stimulation is not satisfactorily understood.

In studying the endogenous calcium and phosphorus exchange during pregnancy it is important to consider the distribution and fixation of these elements not only in the fetus itself but also in the fetal adnexa and the maternal increment. The retention of calcium and phosphorus is necessary for the formation of all additional tissues accompanying fetal development. Mineral elements are utilized in the formation of the placenta and amniotic fluid (14) in response to the requirements of the growing fetus. The normal size of the placenta is usually about one-eighth that of the infant's weight and its ash analysis shows large amounts of both calcium and phosphorus in association with other mineral elements (15). Adair (16) states that the fetus and placenta at term contain about 45 gm. of phosphorus. The fetal adnexa constitute approximately a 3 kilo gain in active tissue growth, according to Harding (4). After conception, an enlargement of the uterus, and of the pelvic, abdominal, and gluteal muscles occurs to prepare the maternal body for labor. The uterus increases enormously in size as pregnancy advances, growing from 30 gm. to approximately 1 kilo in weight (4, 17). Wilson (18), Hoffström (1), and Landsberg (8) have demonstrated, by means

of metabolic balance determinations, that the maternal body generally benefits by laying down new tissue reserves, some of which they believe are in preparation for lactation. Harding (4) contends, however, that a portion of these reserves is lost during the puerperal period, and that they are therefore not available, in their entirety, for the elaboration of milk. The mammæ markedly increase in size before lactation. There is a general building up of protein and fat reserves throughout the maternal body and even the glands of internal secretion hypertrophy with advancing gestation (4). With these compensatory changes, there is not only an actual increase in storage of calcium and phosphorus in the form of active body tissue, but there may be, as well, an alteration in the metabolism of these elements.

Deposits have been noted during pregnancy. Williams (19) points out that calcium depositions have been observed in the skull of the gravid woman. Lastly there is the laying down of stable and labile calcium and phosphorus reserves in the maternal bony and soft tissues subsequent to parturition and lactation. In a remarkable series of studies, Bauer, Aub, and Albright (20) have demonstrated, by animal experiments, that easily mobilizable calcium is not laid down in the shaft of adult animals but in the trabeculæ of bone. They produce convincing evidence to show that "the bone trabeculæ therefore serve as the storehouse of readily available calcium," and that "the shafts have a slow progressive exchange of inorganic salts and are not influenced except in the case of unusual body demands." A negative calcium balance decreases the trabeculæ and a positive balance increases them, which would indicate that the trabeculæ serve as the labile supply of calcium and the cortex as the stable supply. Bauer *et al.* further state that:

"It does not seem unlikely that when the trabeculæ are depleted, the structural part of the skeleton meets demands for calcium, just as in the case of the structural proteins during starvation. But the bone shafts are extremely important in maintaining the body structure. Protected from depletion by this storehouse of readily available calcium, it is only in cases of great need that they are called upon to relinquish calcium. Thus, when the trabeculæ are not present to meet the demand for calcium to form new structural units during growth or stress, it is quite possible that calcium is drawn from the bone shafts, and rickets or osteomalacia results. Such prolonged demand is not common but the appearance of osteomalacia after repeated pregnancies or Graves' disease is very suggestive evidence."

Alterations in the deposition and mobilization of calcium and phosphorus in the maternal organism may be brought about by a variety of factors other than those from the products of conception Determinations of calcium and phosphorus intake and outgo may also be used as a measure of some of these influences. This method measures the quantities of these elements retained by the body. However, it does not distinguish between the substances absorbed in the upper intestinal tract and excreted in the colon from those unabsorbed. An enumeration of additional factors that contribute to or deduct from the bodily supply of calcium may assist in making a more comprehensive interpretation of the metabolic balances herein recorded.

The amount of calcium in the diet is often a limiting factor in the mineral balance. On the other hand, in spite of an adequate supply of minerals in the diet, the acid-base equilibrium of the body and the acid-base properties of the food have their influence upon the retention or paths of excretion of calcium, thus determining the positivity or negativity of the balance. Shohl (3), in discussing the acid-base equilibrium of the body, states that the use of alkaline diets is strongly indicated in pregnancy and lactation. It appears that the body is capable of more sustained effort in maintaining equilibrium on such diets than on acid diets. Although the acid-base value of the food intake fluctuates from day to day in the average dietary, it is estimated that "pregnancy and lactation require additional alkali—a minimum of 150 cc. 0.1 N base per day."

The ingestion of mineral acids (21–24) and diets containing a predominance of acid over base radicals increases calcium excretions (25, 26), thus producing a negative calcium balance. An excess alkali in the diet produces a similar effect (27). Bauer, Albright, and Aub (28) state that negative nitrogen balance may decrease calcium utilization through the production of acid radicals by tissue catabolism. In addition it has been shown that for optimal utilization of calcium from the diet, not only must there be satisfactory acid-base equilibrium with a requisite proportion of food elements (26, 29), but also certain calcium fixation substances must be provided. Vitamins C and D and sunshine (30–34) are known to favor calcium assimilation and utilization by decreasing its excretion. Over and above these factors, the hyperfunctioning

of particular glands of internal secretion favors calcium excretion in excess of the normal (35, 36). Some of these calcifying, decalcifying, and anticalcifying influences may obviously reflect themselves not only in the maternal teeth but also in the teeth and bony structure of the offspring (37–40). Hamilton and Moriarty (29, 41), in their studies draw attention to the fact that the composition of the tissues gained by growth after birth is different from the composition of the body at birth, the former being richer in calcium and phosphorus. Furthermore they point out that:

> "Neither the gain in weight nor the retention of mineral substances during a period of a few days would represent the average gain in weight and the average retention of that infant. It seems probable that the gain in weight on different days is of different quality, sometimes consisting chiefly of fluid, at other times to a greater extent of fat and muscle tissue."

These factors are likewise applicable and of similar importance in the interpretation of the metabolic balances of the pregnant woman. Therefore studies taken over a short period of a few days and only at intervals cannot be considered to indicate a constant typical growth of bone of the fetus or soft tissue and fluid of either the maternal body or fetus.

Little is known regarding the effect of activity upon the calcium and phosphorus balances in man though the matter is under consideration by Bauer and coworkers (28). Turner and Hartman (42), however, studied the effect of activity upon the calcium and phosphorus balances in dairy cows and found it insignificant.

The effect of the psychic state upon calcium and phosphorus retention has not been studied in man, though there are positive indications in the composition of the blood during mental disturbances (43). Meigs and coworkers (44) obtained results which suggested to them that disturbances in the routine treatment of the cow interfere with calcium assimilation. Forbes and coworkers (45) believe that this interpretation is not warranted. Hart *et al.* (46), however, found it necessary to use "quiet animals with phlegmatic temperaments" in metabolic studies.

In conclusion, there are many apparent and variable biological processes operating during the period of pregnancy; their influence is known to be far reaching and inadequately understood. In all

probability there are many yet unrecognized factors significant in the metabolism of women during the reproductive cycle. It is the accumulation and recording of many and diverse data on the same individual and on different individuals in various stages of gestation that will ultimately reveal the functions and physiological processes peculiar to the progression of the human reproductive cycle. The following data concerning calcium and phosphorus balances during human pregnancy are presented with a hope that they will help to bridge some of the gaps in this little explored field.

EXPERIMENTAL.

Procedure.

Three housewives (Table I) whose physiological processes were known to be successfully adapted to secreting large quantities of milk of excellent quality were observed for their calcium and phosphorus metabolism at 4 week intervals during the last half of their pregnancy periods. Although there had been little opportunity for sexual rest since the foregoing reproductive cycles, Subjects VI and VII were known to be storing appreciable quantities of calcium and phosphorus at the 60th and 50th weeks respectively of the lactation period immediately preceding the conception under discussion. During the metabolic balance studies the women participated in their usual activities in the care of their homes and children. The customary diets known to be typical in quantity and quality of those habitually chosen by the respective families were consumed by the women during the metabolic balance studies (47). Distilled water was used, however, *ad libitum* and in tea and coffee.

The collections of all samples of food, feces, and urine for the chemical analyses in the metabolic balance studies were made over a 4 day period in the home. One-half by weight of the voluntary food intake of the individual women was pooled for the daily food composite. After being thoroughly mixed these samples were dried, pulverized, and preserved in air-tight containers. Daily aliquot samples of the milk that was used as a beverage were pooled for the period and analyzed apart from the other food materials. In a similar manner aliquots of the 24 hour specimen of urine were removed and combined to make up the 4 day composite for the determination of the calcium and phosphorus

TABLE I.

Case Histories of Subjects.

	Subject VI.	Subject VII.	Subject VIII
Descent.	Dutch, French, German, Danish.	Scotch, Irish, American.	Dutch, French, German, Danish.
Age, *yrs.*	25	33	27
Height, *in.*	62.2	68.5	62.6
Body weight, *lbs.*	140	135	150
Usual physical state.	Apparently healthy, persistently low hemoglobin.	Healthy, no defects.	Apparently healthy, albuminuria.
Basal metabolic rate.	28 per cent +.	2.7 per cent +.	14.5 per cent +.
Reproductive History.			
No. of pregnancies.	4	3	3
Health during pregnancies.	Good.	Excellent.	Good.
Development of infant.	Excellent.	"	Excellent.
Duration of preceding lactation, *wks.*	60	50	50
Range of 24 hr. milk production, *oz.*	77–113	21–90	13–50
Interval to conception.	None.	1 mo.	1 mo.
Present reproductive cycle.			
Last menstrual period.	Nov. 10, 1927.	Nov. 18, 1927.	Oct. 29, 1927.
First fetal movement.	Apr. 1, 1928.	Apr. 8, 1928.	Mar. 15, 1928.
Health.	Nausea first trimester.	Excellent.	Apparently good.
Routine urine examination.	Negative.	Negative.	Albumin ++++.
Total gain in weight, *lbs.*	51½	26½	27
Duration of pregnancy, *wks.*	40	43	38
Date of parturition.	Aug. 20, 1928.	Sept. 14, 1928.	July 17, 1928.
Character of labor.	Normal, long.	Normal, short.	Normal, short.
Weight of infant.	8 lbs., 10 oz.	9 lbs., 3 oz.	6 lbs., 12 oz.
State of mother in puerperium.	Excellent.	Excellent.	Good.
Quantity of milk.	Abundant.	Abundant.	Abundant.

XUM

excreted in the urine during the metabolic balance study. The feces for the period were marked off by carmine, treated with hydrochloric acid and alcohol, dried, ground, and kept in jars until analyzed.

The food, milk, urine, and feces were ashed in platinum crucibles at approximately 500°, after which they were dissolved in hydrochloric acid. The interfering silicates were removed by treating the ash of the food and feces with hydrochloric acid and heat as recommended by Clark (48). All ash samples were made up to volume and aliquot portions taken for the gravimetric analyses. The calcium in all cases was determined by the modified McCrudden method (49); the phosphorus in the foods and feces by the McCandless and Burton method (50) and that in the urine and milk by the Epperson method (51).

Systematic detailed psychological observations on the women accompanied all metabolic balance studies. The method of studying and recording the emotional reactions has been reported elsewhere (52).

Roentgenograms of the teeth and complete oral examinations were made in early pregnancy and at its termination. Roentgenograms of the long bones of the infants were made on the 10th and 42nd days after birth.

RESULTS AND DISCUSSION.

The data of the calcium and phosphorus balances for three women in the last half of pregnancy are given in Tables II and III. The daily results are presented in units of gm. of minerals per kilo of body weight and in total gm. in order to facilitate comparison among our subjects and with other available data. Appreciable losses of calcium were experienced by Subject VI at the 26th and 30th weeks of the gestation period, by Subject VII at the 14th week, and by Subject VIII at the 30th and 34th weeks. In only two instances did the negative phosphorus retentions parallel those of the calcium and these occurred for Subject VI in the 26th week of pregnancy and for Subject VIII in the 34th week. The storage of phosphorus may indicate the laying down of soft body tissue.

The total number of gm. of calcium and phosphorus in the daily food intake, together with the outgo in the urine and feces, is

TABLE II.

Calcium Retention in Pregnancy.

The results are expressed in gm. per day and per kilo of body weight per day.

Subject No.	Wk. of pregnancy.	Body weight.	Food consumption.						Excretion.						Retention.	
			Solid food.		Milk.		Total intake.		Urine.		Feces.		Total outgo.			
			Day.	Kilo.	Day.	Kilo.	Day.	Kilo.	Day.	Kilo.	Day.	Kilo.	Day.	Kilo.	Day.	Kilo.
		kg.														
VI	20	74.7	0.613	0.008	2.080	0.028	2.693	0.036	0.420	0.006	2.211	0.030	2.631	0.035	+0.062	+0.0008
	26	80.6	0.531	0.007	1.462	0.018	1.993	0.025	0.489	0.006	2.780	0.034	3.269	0.040	−1.276	−0.0158
	30	84.2	0.544	0.006	1.641	0.020	2.185	0.026	0.697	0.008	1.856	0.022	2.553	0.030	−0.368	−0.0042
	34	86.5	0.698	0.008	1.081	0.013	1.779	0.021	0.635	0.007	0.978	0.011	1.613	0.018	+0.166	+0.0019
	38	86.9	0.709	0.008	1.083	0.013	1.792	0.021	0.669	0.008	1.067	0.012	1.736	0.020	+0.056	+0.0007
VII	14	66.3	0.683	0.010	1.011	0.015	1.694	0.025	0.524	0.008	1.390	0.021	1.914	0.029	−0.220	−0.0033
	26	72.1	0.935	0.013	0.986	0.014	1.921	0.027	0.501	0.007	1.235	0.017	1.736	0.024	+0.185	+0.0027
	30	73.1	0.759	0.010	0.949	0.013	1.708	0.023	0.431	0.006	0.999	0.014	1.430	0.020	+0.278	+0.0038
	34	75.0	0.851	0.011	0.910	0.012	1.761	0.023	0.525	0.007	1.073	0.014	1.598	0.021	+0.163	+0.0022
	38	75.6	0.753	0.010	0.847	0.011	1.600	0.021	0.404	0.005	0.962	0.013	1.366	0.018	+0.234	+0.0032
VIII	30	79.2	0.754	0.010	1.656	0.021	2.410	0.031	0.263	0.003	2.204	0.028	2.467	0.031	−0.057	−0.0007
	34	79.8	0.406	0.005	1.125	0.014	1.531	0.019	0.166	0.002	1.830	0.023	1.996	0.025	−0.465	−0.0060

TABLE III.

Phosphorus Retention in Pregnancy.

The results are expressed in gm. per day and per kilo of body weight per day.

Subject No.	Wk. of pregnancy.	Body weight.	Food consumption.						Excretion.						Retention.	
			Solid food.		Milk.		Total intake.		Urine.		Feces.		Total outgo.			
			Day.	Kilo.	Day.	Kilo.	Day.	Kilo.	Day.	Kilo.	Day.	Kilo.	Day.	Kilo.	Day.	Kilo.
		kg.														
VI	20	74.7	1.492	0.020	1.474	0.020	2.966	0.040	1.580	0.021	0.858	0.011	2.438	0.033	+0.528	+0.0071
	26	80.6	1.335	0.017	1.047	0.013	2.382	0.030	1.442	0.018	1.102	0.014	2.544	0.032	−0.162	−0.0020
	30	84.2	1.381	0.016	1.472	0.018	2.853	0.034	1.705	0.020	0.616	0.007	2.321	0.027	+0.532	+0.0063
	34	86.5	1.400	0.016	0.772	0.009	2.172	0.025	1.496	0.017	0.414	0.005	1.910	0.022	+0.262	+0.0030
	38	86.9	1.150	0.013	0.774	0.009	1.924	0.022	1.003	0.012	0.462	0.005	1.465	0.017	+0.459	+0.0053
VII	14	66.3	1.369	0.021	0.685	0.010	2.054	0.031	1.019	0.015	0.910	0.014	1.929	0.029	+0.125	+0.0008
	26	72.1	2.137	0.030	0.615	0.009	2.752	0.039	1.108	0.015	0.932	0.013	2.040	0.028	+0.712	+0.0099
	30	73.1	1.594	0.022	0.676	0.009	2.270	0.031	1.396	0.019	0.810	0.011	2.206	0.030	+0.064	+0.0010
	34	75.0	1.516	0.020	0.648	0.009	2.164	0.029	1.222	0.016	0.807	0.011	2.029	0.027	+0.135	+0.0018
	38	75.6	1.443	0.019	0.593	0.008	2.036	0.027	0.836	0.011	0.732	0.010	1.568	0.021	+0.468	+0.0063
VIII	30	79.2	0.890	0.011	1.051	0.013	1.941	0.024	1.217	0.015	0.688	0.009	1.905	0.024	+0.036	+0.0005
	34	79.8	0.679	0.008	0.780	0.010	1.459	0.018	0.873	0.011	0.634	0.008	1.507	0.019	−0.048	−0.0010

illustrated with their resultant balances in Charts I and II. The large amounts of calcium and phosphorus consumed in the form of milk are also indicated in Tables II and III. It is of significance to note that these intakes are in excess of the present recommended food requirements for maternity (53, 54). For all three subjects there was a daily fluctuation of calories from 2400 to 3600, of nitrogen from 11.6 to 23.6 gm., of calcium from 1.5 to 2.7 gm., and of phosphorus from 1.5 to 3.0 gm.

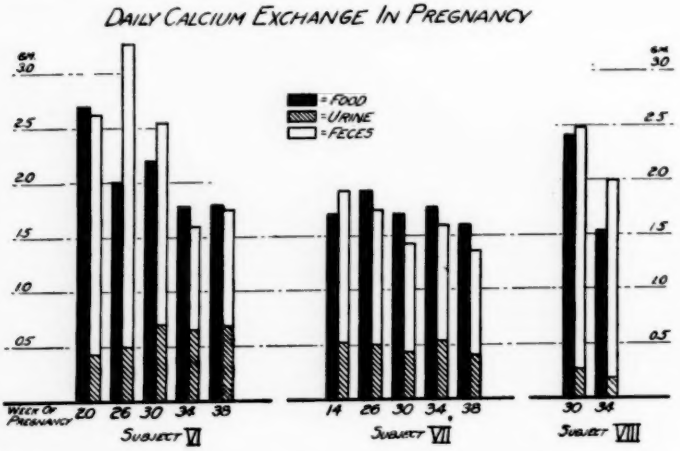

CHART I. Daily calcium exchange in pregnancy. The solid black columns illustrate the total number of gm. of calcium in the daily food intake; the plain and shaded columns show the total outgo of calcium in the feces and urine, respectively. The difference between the height of the intake and outgo columns represents the calcium balance. When the solid column exceeds the outgo, calcium is being stored in the body; on the other hand, when the opposite condition exists, calcium is being lost.

In spite of the large consumption of calcium and phosphorus, excessive excretion occurred. Although the diets were undirected, there was an excess of base over acid, these values varying from 4 to 30 cc. of 0.1 N base when the diets were calculated from the analyses of food given by Sherman and Gettler (55). The figures for the entire periods under consideration obscure the daily fluctuations in the acid-base properties of the diet. On the

whole, however, it would appear that the diets of our subjects have a tendency towards the alkaline which is in accordance with the recommendations suggested by Shohl (3) for pregnancy. Since a negative nitrogen balance may influence the acid-base equilibrium of the body by adding acid radicals from tissue catabolism (28), it is relevant to know that only one negative nitrogen balance was observed and that was found in Subject

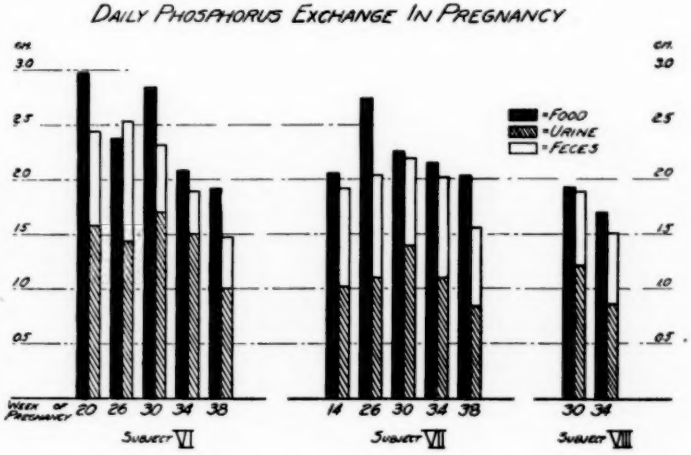

CHART II. Daily phosphorus exchange in pregnancy. The solid black columns illustrate the total number of gm. of phosphorus in the daily food intake; the plain and shaded columns show the total outgo of phosphorus in the feces and urine, respectively. The difference between the height of the intake and outgo columns represents the phosphorus balance. When the solid column exceeds the outgo, phosphorus is being stored in the body; on the other hand, when the opposite condition exists, phosphorus is being lost.

VIII in her 34th week of pregnancy. A more complete discussion of this phase of metabolism will appear in a later publication on the nitrogen balance and the nitrogen partition of the urine as they are influenced by conception and pregnancy.

The Ca : P ratios of the food, excreta, and retentions are recorded in Table IV. These data must be considered not only in the

proportion for the formation of fetal bone but for the formation of fetal and maternal tissue as well.

TABLE IV.

Calcium-Phosphorus Ratios in Pregnancy Balances.

Subject No.	Wk. of pregnancy.	Food.	Urine.	Feces.	Retention.
VI	20	0.91	0.27	2.58	0.12
	26	0.84	0.34	2.52	*
	30	0.77	0.41	3.01	*
	34	0.82	0.42	2.36	0.63
	38	0.93	0.67	2.31	0.12
VII	14	0.82	0.51	1.53	*
	26	0.70	0.45	1.33	0.26
	30	0.75	0.31	1.23	4.34
	34	0.81	0.43	1.33	1.21
	38	0.79	0.48	1.31	0.50
VIII	30	1.24	0.22	3.20	*
	34	1.05	0.19	2.89	*

* Negative balances were found.

TABLE V.

Paths of Excretion of Calcium and Phosphorus in Pregnancy.

Subject No.	Wk. of pregnancy.	Calcium.				Phosphorus.			
		Per cent of intake.		Per cent of outgo.		Per cent of intake.		Per cent of outgo.	
		Urine.	Feces.	Urine.	Feces.	Urine.	Feces.	Urine.	Feces.
VI	20	15.6	82.1	16.0	84.0	53.3	28.9	64.8	35.2
	26	24.5	139.5	15.0	85.0	60.5	46.3	56.7	43.3
	30	31.9	84.9	27.3	72.7	59.8	21.6	73.5	26.5
	34	35.7	55.0	39.4	60.6	68.9	19.1	78.3	21.7
	38	37.3	59.5	38.5	61.5	52.1	24.0	68.5	31.5
VII	14	30.9	82.0	27.4	72.6	49.6	44.3	52.8	47.2
	26	26.1	64.3	28.9	71.1	40.3	33.9	54.3	45.7
	30	25.2	58.5	30.1	69.9	61.5	35.7	63.3	36.7
	34	29.8	60.9	32.9	67.1	56.5	37.3	60.2	39.8
	38	25.3	60.1	29.6	70.4	41.1	36.0	53.3	46.7
VIII	30	10.9	91.5	10.7	89.3	62.8	35.5	63.9	36.1
	34	10.8	119.5	8.3	91.7	59.6	43.4	57.9	42.1

The percentage of total intake excreted and the percentage of total outgo in urine and feces are shown in Table V. The paths of excretion are illustrated in Charts I and II. In discussing the fluctuations of calcium metabolism, Bauer and coworkers (28) state that:

"there is considerable variation in the calcium excretion not only among different individuals but also among different periods of the same individual. This variation could probably be reduced by a more rigid standardization of the aforementioned normal factors [sex, age, weight or body surface, activity, acid-base values of diet, amounts of other cations in intake, and phosphorus metabolism] which influence calcium metabolism."

These authors studied a gravid woman and found that her calcium excretion was essentially normal. Their data tend, however, to show that calcium excreted on a low calcium intake was not available for the fetus. These results are similar to those of Hamilton and Moriarty (29), who showed that it was not available for bone building during growth.

In view of the fact that the calcium and phosphorus balances may be affected by sunshine (31, 34), it is relevant to state that parturition took place in all three subjects between July and September. If there is a seasonal influence, then, all three women would be similarly affected. Furthermore, since their habits of living are similar, hygiene cannot be said to be responsible for individual differences.

Little is known about the effect of activity upon the calcium and phosphorus metabolism in human beings. In this study the amount of sleep and activity of the women have been recorded but the data are not included in this report, since there is no means of interpreting them quantitatively.

The effect of the mental state upon the physiological processes is indicated. In view of this relationship, it is appropriate to call attention to the fact that the psychological and emotional records verified the fact that Subject VI was undergoing great worry and anxiety during the 26th week, a period in which she gave the greatest negative calcium balance. These findings would tend to substantiate the observations of Meigs, Blatherwick, and Cary (44) and Hart, Steenbock, Scott, and Humphrey (46) in their study of the calcium and phosphorus metabolism in the cow.

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The products of conception (4) and activity and emotion (56) stimulate the glands of internal secretion to hyperfunction. The augmented activity of the endocrine system during pregnancy complicates the problem of interpreting the calcium and phosphorus balance.

Quantitative methods for recording and rating the oral conditions and changes in the mouths of the women have not yet been elaborated. There is a common opinion that weakening of the maternal bones and teeth frequently accompanies pregnancy owing to the withdrawal of calcium. From his observations on gravid women, Sorrin (57) states that, "When the nutritional requirements are neglected, a gingivitis, followed by loss of bone is observed. Often caries, extreme sensitiveness and local acidity develop."

It is apparent from these data on three pregnant women who were living in their usual home environment and eating their accustomed dietaries that there is a noticeable variation in the metabolism among the individuals as well as in the same woman at different times. These data illustrate the status of the calcium and phosphorus metabolism during 4 day studies at 4 week intervals in the last half of the gestation period, values that may be considered, for these subjects, typical of the specific period in the physiological response accompanying the progression of gestation. With the paucity of available information relating to the effect of frequent pregnancies followed by a long and abundant milk flow, it is difficult to predict what biological processes are involved in the maintenance of the calcium and phosphorus reserves in the human organism. It is further evident, from the facts presented in this report, that our knowledge at present does not warrant any precise interpretations in regard to the physiological mechanism involved in the calcium and phosphorus metabolism of the pregnant woman or of her requirements during this crucial period. The results from this limited group of exceptionally well endowed gravid women are governed by the particular physiological activities of the individuals and are therefore significant only as they contribute to our sum total of knowledge of the metabolism of women during the reproductive cycle.

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SUMMARY.

1. Records of calcium and phosphorus balances of three women who were consuming their usual home diets, known to be abundant in food essentials, were made at monthly intervals during the last half of pregnancy.

2. Preceding conception all three women had completed a year of heavy milk flow.

3. Appreciable losses of calcium were experienced by Subject VI at the 26th and 30th weeks of the gestation period, by Subject VII at the 14th week, and by Subject VIII at the 30th and 34th weeks.

4. Phosphorus was retained in large amounts at the periods observed, with but two exceptions, by all three subjects.

5. There were noticeable variations in the metabolism of different individuals at the same time in the gestation period, as well as in the same individual at different times during the period.

6. This report shows a practical need for scientific information concerning the calcium and phosphorus metabolism of women living in their usual home environments and consuming their accustomed diets during the reproductive cycle.

It is a pleasure to express our appreciation for the continued interest and cooperation of the three women who have served so faithfully and loyally as subjects for this investigation and to express our thanks to their personal physicians and dentists for their helpful advice and cooperation.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE.

II. CALCIUM AND PHOSPHORUS UTILIZATION IN TWO SUCCESSIVE LACTATION PERIODS.*

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It is known that maternal physiological responsibility does not terminate with parturition but proceeds in an even more intense degree during lactation. In order to interpret metabolic findings during the progression of lactation, it is important to know what chemical events have taken place in the maternal body during the preceding pregnancy and lactation periods. Accordingly the unit in a study of the metabolism of reproduction comprises both pregnancy and lactation (1). The purpose of the present report is to record the calcium and phosphorus utilization in women during early, mid-, and late lactation.

It has been demonstrated that there are great demands for

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† The data are taken from the dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Ogden Graduate School of Science in the University of Chicago. The work was done under the tenure of fellowships of the Merrill-Palmer School in Nutrition Research in 1925–26; of the National Research Council in Child Development and Parent Training under a grant of the Spelman Fund in 1927–28; and of the National Research Council in Child Development under a grant of the Spelman Fund in 1929–30.

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calcium and phosphorus during pregnancy, not only for the development of the fetus and its adnexa but also for maternal reserves in preparation for labor and lactation (2, 3). Furthermore, the literature presents convincing evidence of losses in these elements in ordinarily well fed lactating animals. All these findings suggest the significance of studies of calcium and phosphorus metabolism during the entire reproductive cycle of women.

HISTORICAL.

No studies of the mineral metabolism of lactating women have been available to the writer. In view of this lack of material, it may be of value to review briefly some of the literature dealing with certain phases of the mineral metabolism of lactating animals.

Effect of Continuous Sexual Activity.—It is not known what effects are brought about in the physiological processes of woman by frequent repetitions of the reproductive cycle, especially when the gestation period is followed by a long and abundant milk flow. Orr and Magee (4) have found that the absence of sexual rest in the cow not only leads to a loss of calcium and phosphorus from the maternal body but also produces a metabolic fatigue in the absorptive function and in the assimilation of these elements.

Effect of Lactation on Bones.—Macomber (5) found that the withdrawal of calcium from the bodies of rats on a calcium-low diet, during pregnancy, was intensified during lactation. Sherman and MacLeod and Sherman and Quinn (6, 7) found that there was an appreciable loss of calcium and phosphorus from the bodies of female rats that had borne and nourished young. During pregnancy and lactation these animals had been fed on diets rich in calcium and phosphorus beyond all requirements of the most rapid growth.

Factors Influencing Calcium and Phosphorus Balances in Lactating Animals.

Stages of Lactation.—Several workers (8–13) have shown that the goat and cow are unable to maintain positive calcium and phosphorus balances in early lactation in spite of high consumption of these elements. That there is, however, a storage of calcium and phosphorus in both animals in late lactation, when the quantity of milk decreases so that the powers of assimilation can meet the demands of the milk-secreting impulse, has been demonstrated by Forbes *et al.* (8) and Miller, Yates, Jones, and Brandt (14) for cows, and by Hunt, Winter, Schultz, and Miller (12) for goats.

Levels of Intake.—Forbes *et al.* (8), Hart, Steenbock, Hoppert, Bethke, and Humphrey (15), and Turner and Hartman (13) found that the addition of mineral supplements to the diet failed to establish positive calcium and phosphorus balances, or even equilibrium. On the other hand, high levels of these supplements produced positive retentions in experimental animals observed later by Hart and his associates (16), Miller *et al.* (11), and Hunt *et al.* (12).

Ca : P Ratio in Food.—Meigs and coworkers (17) have placed the optimal value of the Ca: P ratio for lactating cows at 1.0 to 1.5. The results of their studies of the calcium and phosphorus balances indicated to them that phosphorus assimilation might be interfered with by an excess of calcium, and that 2 parts by weight of calcium to 1 of phosphorus represented an excess. Hart *et al.* (18) found positive balances on ratios of 1.1 to 1.2 in the food, but believed that a definite ratio was not as important as the provision of a liberal plane of each element in the presence of an ample supply of the antirachitic vitamin.

Acid-Base Equilibrium.—Miller and his associates (14) found that introducing bone meal or kale into the ration of cows caused a shift from a negative base balance toward a positive, and on the basis of these results concluded that absorption from the intestine or secretion into the intestine appeared to be the regulatory mechanism in better assimilation of calcium and phosphorus.

Vitamin C.—Hart and coworkers (19) found that orange juice and raw cabbage did not favorably affect calcium assimilation, and concluded that vitamin C was not a factor in assimilation of calcium. On the other hand, Miller *et al.* (14) were able to show improvement in retention in both calcium and phosphorus when cows were fed green kale. That calcium and phosphorus equilibrium could be maintained in milking animals when liberal amounts of these elements were accompanied by green roughage was shown by Turner *et al.* (20), Miller and coworkers (14), Hart and his associates (15, 18, 21), and Hunt *et al.* (12).

Ultra-Violet Rays.—Hart and his coworkers (21–23) found no favorable effects on calcium assimilation in cows exposed to sunlight or the quartz mercury lamp. These workers (24, 25), however, as well as Henderson and Magee (26), found that the exposure of goats to ultra-violet light had a favorable effect upon the assimilation of calcium by reducing its excretion in the feces. The results on phosphorus assimilation were variable.

Cod Liver Oil.—Meigs, Turner, Harding, Hartman, and Grant (17) found no improvement in the utilization of calcium in the dairy cow when cod liver oil was administered. Harvey (27) and Hart and his associates (19, 28), however, were able to induce positive calcium balances in goats, with variable results in phosphorus retention. The latter workers believe this is convincing evidence that the antirachitic factor of cod liver oil is as effective an agent in promoting calcium assimilation in lactating animals as in growing animals.

From the above review, it would seem that the depleted mineral reserves in lactating animals of some species, evidenced by the negative calcium and phosphorus balances, can be restored to some degree by feeding a generous supply of calcium and phosphorus and by providing the antiricketic factor in green feeds, cod liver oil, or ultra-violet light.

EXPERIMENTAL.

Studies were conducted on the calcium and phosphorus retention in three women who were producing large quantities of milk and voluntarily ingesting high levels of these elements in food. The balances were determined in early, mid-, and late lactation in order to observe some of the physiological activities occurring during lactation under these conditions.

General Procedure.

Three American multiparæ, superior milk producers in the Mother's Milk Bureau of Detroit, were selected as subjects for this investigation. Medical examination[1] shows these women to be well, except that one, who gives no evidence of inferior health, has shown albuminuria.[2] These women realized the importance of accuracy in the studies and have at all times exhibited unusual cooperation and interest.

Preliminary to and continuous with the balance studies reported in this paper, chemical studies of the breast milk of these women were made on 24 hour samples at monthly intervals from the 6th week after parturition until the cessation of lactation. The milk of two women, Subjects VI and VII, has been observed throughout two successive lactation cycles, Cycles A and B, and of the third, Subject VIII, during one cycle, Cycle B. The weight of the 24 hour samples of breast milk and the total amounts of calcium and phosphorus elaborated daily in the milk as lactation progressed, are shown in Table I. The extensive chemical study of human milk will be reported in detail in subsequent papers by the staff of the Nutrition Research Laboratories of the Merrill-Palmer School and the Children's Hospital of Michigan.

The phenomenal mineral exchange observed in the chemical studies of the individual milks suggested the importance of metabolic balances, and led, in the fall of 1927, to the making of 10 day balance studies during the 60th and 50th weeks, respectively, of lactation Cycle A in Subjects VI and VII, when both women chose to cease lactating.

[1] The women producers of the Mother's Milk Bureau of Detroit are examined and approved by a member of the staff of the Woman's Hospital.

[2] The writer is indebted to A. S. Guimaraes, M.D., for his interest and cooperation.

Pregnancy followed almost immediately the cessation of lactation in these subjects. The metabolic findings for this period have been reported in full in a previous publication (1). Observations were made simultaneously on a third woman who was in approximately the same period of gestation as Subjects VI and VII.

At the 7th week of the subsequent lactation, Cycle B, the bal-

TABLE I.

Quantity of Milk, Calcium, and Phosphorus Secreted by Women; Study of 24 Hour Samples of Milk Collected during Specific Weeks of Two Successive Lactation Cycles.

Subject No.	Wk. of lactation.....	7	11	15	19	23	27	31	35	39	43
	Lactation Cycle A (1926–27).										
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
VI	Milk.....	3159	3148	3415	3310	3227	3154	2907		2896	2325
	Ca.....	1.02	1.10	1.20	1.06	1.09	1.06	0.94		0.91	0.68
	P.....	0.43	0.41	0.44	0.40	0.44	0.40	0.35		0.33	0.28
VII	Milk.....	2737	2719	2740	2453	2574	2491		1450	629	
	Ca.....	0.80	0.80	0.79	0.72	0.66	0.76		0.35	0.15	
	P.....	0.40	0.41	0.39	0.36	0.40	0.40		0.21	0.10	
	Lactation Cycle B (1928–29).										
VI	Milk.....	3267	3643	3560	3488	3659	3566				
	Ca.....	1.16	1.24	1.26	1.24	1.58	1.32				
	P.....	0.46	0.43	0.37	0.34	0.39	0.39				
VII	Milk.....	2588	2734	2630	2799	2521	2524				
	Ca.....	0.75	0.87	0.85	0.79	0.82	0.83				
	P.....	0.43	0.40	0.40	0.38	0.37	0.39				
VIII	Milk.....	1395		1516	1592	1600	1620				
	Ca.....	0.41		0.59	0.54	0.47	0.47				
	P.....	0.23		0.23		0.20	0.22				

ances of the three women were determined. Clinical observation indicates that, in general, the maternal organism has by this time made complete physiological readjustment from the states of pregnancy and parturition to that of lactation.

Metabolic balances of the mid-stage of lactation were determined at the 27th week, when the quantity of milk produced

(Table I) indicated that the women were maintaining their highest plateau of secretion, and that a bodily mineral depletion might therefore result. The balances were determined on the basis of 4 day collection periods in the early and mid-stages of lactation, during the fall and winter of 1928–29.

The women lived at home and carried on their usual duties under the observation of the writer, who lived there during the studies, making the collections of breast milk, food, urine, and feces for chemical analysis in the laboratory.

Collection of Food.—All food and liquids taken by the women during the balance periods were weighed. Aliquot portions of one-half by weight of the food ingested were taken for the daily composite in the chemical studies. After the addition of hydrochloric acid and alcohol, these samples were placed in a steam oven and dried at a temperature below 100°. When constant weight was secured, the dried samples were pulverized finely, blended, and preserved in air-tight containers. The composite samples for the balance periods were made by thoroughly mixing aliquot portions of each day's dried food sample.

The large quantities of cow's milk used as a beverage made it impossible to include it with the samples of food for drying, and consequently a composite of milk from each day's intake was made for the balance period and was analyzed separately. When butter was used, the calcium and phosphorus contents were computed from values given by Sherman (29), since it was thought that possible variations in the elements found in analysis would have no appreciable effect on the results. Distilled water was used *ad libitum* for drinking and in tea and coffee.

Dietary Studies.—At the time of analysis of the monthly samples of milk during lactation Cycles A and B, quantitative studies of the voluntary food intake of the women were made by weighing the individual foods and computing their values by the use of Sherman's (29) figures for the composition of foods. Considerable data have been collected concerning both the quantity and quality of the individual diets. Frequent visits of the observer in the homes over $2\frac{1}{2}$ years have confirmed the similarity of the diets observed to those habitually consumed there.

Calculated dietary studies were made during the metabolism balance period to determine the distribution of calcium and phos-

phorus in the diets of the women, since these data could not be secured from the chemically analyzed composite sample of food. The amount and the percentage of the elements from various food sources in the voluntarily chosen food intakes, such as fruits,

TABLE II.

Distribution of Calcium and Phosphorus in Accustomed Diets.

The results are expressed in gm. and per cent of the total computed intake.

Subject No.	Wk. of lactation.	Fruit.		Vegetable.		Cereal.		Egg and cheese.		Meat.		Milk.		Total.
						Calcium.								
		gm.	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*
VI	60	0.11	4	0.12	4	0.09	3	0.25	9	0.04	1	2.08	78	2.92
	7	0.08	3	0.18	7	0.06	2	0.41	16	0.02	1	1.83	71	2.60
	27	0.08	2	0.14	4	0.09	3	0.43	13	0.03	1	2.51	77	3.28
VII	50	0.07	2	0.13	4	0.21	6	0.27	7	0.02	0.5	3.06	81	3.75
	7	0.04	1	0.12	4	0.12	4	0.26	8	0.01	0.3	2.80	84	3.35
	26	0.05	2	0.16	5	0.23	7	0.26	8	0.01	0.4	2.45	78	3.16
VIII	7	0.004	0.1	0.14	4	0.07	2	0.63	17	0.02	0.4	2.85	77	3.71
	27	0.01	0.3	0.12	3	0.08	2	0.38	11	0.01	0.1	2.85	83	3.43
						Phosphorus.								
VI	60	0.11	3	0.32	9	0.28	8	0.35	10	0.57	17	1.78	51	3.43
	7	0.08	3	0.34	11	0.26	9	0.43	15	0.46	15	1.42	48	2.99
	27	0.08	2	0.31	9	0.28	8	0.46	13	0.54	15	1.94	54	3.61
VII	50	0.09	3	0.34	8	0.69	17	0.26	6	0.39	9	2.36	56	4.12
	7	0.06	2	0.32	9	0.47	14	0.22	6	0.21	6	2.17	63	3.44
	26	0.07	2	0.33	9	0.87	24	0.31	8	0.22	6	1.90	51	3.70
VIII	7	0.01	0.2	0.29	8	0.30	8	0.55	15	0.27	8	2.21	61	3.40
	27	0.02	0.5	0.27	8	0.27	8	0.53	16	0.09	3	2.22	65	3.39

vegetables, cereals, cheese and eggs, and meat and milk, are shown in Table II. In all cases milk was the chief source of calcium and phosphorus. It contributed as much as 1.8 to 3.0 gm. of calcium daily, or from 71 to 84 per cent of the total consumption; and 1.4 to 2.4 gm. of phosphorus, which represented from 48 to 65 per cent

of the intake. Subjects VI and VIII ate more eggs and cheese than Subject VII. Subject VIII used very little fruit as compared to Subjects VI and VII. Subject VII had a large consumption of bread and other cereals. Subject VI ate more meat than either Subjects VII or VIII. The differences in the degree of utilization of the elements from these sources of food materials in human milk production are under consideration.

Collection of Urine and Feces.—24 hour collections of urine were made during each metabolism period. The urine was preserved with toluene and kept cold in flasks. The daily quantity was measured, some removed for immediate analysis, and an aliquot portion set aside for the composite sample of the balance period. The latter was used for mineral determinations.

The stools were marked off by carmine at the beginning and at the end of each balance period. The feces were treated with alcohol and hydrochloric acid, dried, ground, and preserved in air-tight jars.

Collection of Breast Milk.—The women expressed their milk by hand at 4 hour intervals during each 24 hour day throughout all the studies. Aliquot portions were taken of these 4 hour samples for the 24 hour composite, and in turn aliquot portions of the daily production were pooled for the balance composite. Ash determinations for calcium and phosphorus were made on each day's milk as well as on the period composite.

Chemical Analyses.—The breast and cow's milk, urine, food, and feces were ashed in platinum crucibles at approximately 500° in an electric muffle furnace. All ashes were dissolved in hydrochloric acid. To remove the interfering silicates in calcium precipitation, the ashes of the foods and feces were treated with hydrochloric acid and heat according to the method described by Clark (30). Calcium was determined in all samples by McCrudden's method (31), but methyl red was used as an indicator for ascertaining the correct pH for the precipitation of calcium oxalate. The calcium was ignited and weighed as calcium oxide. The phosphorus in the aliquot samples of the ashes of the foods and feces was determined by the McCandless and Burton method (32); thus the phosphorus was precipitated as ammonium phosphomolybdate and magnesium ammonium phosphate and weighed as pyrophosphate. All phosphorus analyses of the urine and milk

have been made by the Epperson method (33) of double precipitation of magnesium ammonium phosphate and weighed as pyrophosphate. In the determinations on the breast milk for the first lactation balances, the micro methods were used—Clark's method (34) for calcium in blood, and Fiske and Subbarow's (35) colorimetric method for phosphorus.

RESULTS AND DISCUSSION.

The analyses of the calcium and phosphorus intake and outgo with their consequent daily balances on three women during early, mid-, and late lactation are given in Table III. During the period of high milk production, 7 and 27 weeks after parturition in lactation Cycle B, negative balances prevailed, but when the quantity of milk flow had decreased in the 50th and 60th weeks of the previous lactation Cycle A, positive retentions were found.

The two subjects whose milk production had decreased in the late stage of lactation Cycle A were found in positive calcium retention (Tables I and III). These balance results are in accord with findings of several investigators: Hunt *et al.* (12), working with goats, and Miller *et al.* (14) and Forbes and associates (8), with cows, found the animals capable of storing calcium after "the milk production had decreased to such an extent that calcium outgo did not exceed their capacities to assimilate calcium" (8).

Observations in early lactation showed all three subjects to be in negative calcium balance in spite of the fact that the intake in all cases exceeded the amount secreted in the breast milk. Subject VIII was losing calcium at an excessive rate. 20 weeks later all the women had progressed into a greater negative calcium balance. This state perhaps represents the culminative effect of the maternal loss and the inability of the maternal body to assimilate enough calcium to meet the milk-secreting demand superimposed upon the greatly increased mineral metabolism of gestation. Since the voluntary calcium intake had increased 0.57, 0.47, and 0.80 gm., respectively, for Subjects VI, VII, and VIII between the 7th and 27th weeks of lactation, and since, further, the increased output in breast milk had been only 0.056, 0.135, and 0.053 gm., the negative calcium balance cannot be explained by an inadequate intake of this element. There must be other factors operating to inhibit calcium assimilation.

TABLE III.

Daily Calcium and Phosphorus Balances of Women at Different Weeks of Two Successive Lactation Cycles.

	Subject VI.			Subject VII.			Subject VIII.	
Lactation Cycle	A	B		A	B		B	
Wk. of lactation	60	7	27	50	7	26	7	27
Calcium.								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Intake.								
Solid food	0.962	0.669	0.824	0.937	0.508	0.854	0.878	0.657
Cow's milk	2.004	2.148	2.566	2.983	2.852	2.978	2.742	3.767
Total	2.966	2.817	3.390	3.920	3.360	3.832	3.620	4.424
Outgo.								
Breast milk	0.577	1.239	1.295	0.284	0.706	0.841	0.474	0.527
Urine	0.196	0.075	0.400	0.321	0.071	0.072	0.134	0.126
Feces	1.989	1.923	3.849	2.829	2.637	3.668	4.369	5.612
Total	2.762	3.237	5.544	3.434	3.414	4.581	4.977	6.265
Retention	+0.204	−0.420	−2.154	+0.486	−0.054	−0.749	−1.357	−1.841
Assimilation, *gm.*	+0.781	+0.819	−0.859	+0.770	+0.652	+0.092	−0.883	−1.314
" *per cent*	+26.3	+29.1	−25.3	+19.6	+19.4	+2.4	−24.4	−29.7
Phosphorus.								
Intake.								
Solid food	1.780	1.422	1.765	1.991	1.185	2.029	1.241	1.121
Cow's milk	1.487	1.557	1.845	2.341	1.957	1.554	2.002	2.103
Total	3.267	2.979	3.610	4.332	3.142	3.583	3.243	3.224
Outgo.								

Total	3.267	2.979	3.610	4.332	3.142	3.585	3.243	3.224
Outgo.								
Breast milk	0.252	0.485	0.402	0.199	0.470	0.384	0.263	0.231
Urine	1.621	1.817	2.791	1.782	1.358	0.886	1.820	0.471
Feces	1.011	0.787	1.234	1.558	1.500	1.687	1.186	1.271
Total	2.884	3.089	4.427	3.539	3.328	2.957	3.269	1.973
Retention	+0.383	−0.110	−0.817	+0.793	−0.186	+0.626	−0.026	+1.251
Assimilation, *gm.*	+0.635	+0.375	−0.415	+0.992	+0.284	+1.062	+0.237	+1.482
" *per cent.*	+19.4	+12.6	−11.5	+22.9	+9.0	+29.2	+7.3	+46.0

Among the eight phosphorus balances, four—two in late and two in mid-lactation—were positive (Table III). The two storage periods in mid-lactation were accompanied by increased calcium losses. These results are in harmony with those observed in animals in which, it is thought, phosphorus is more easily obtainable for storage than is calcium because of its more extensive metabolism in soft tissues than in skeletal tissues (8). In these studies the results for phosphorus usually, but not consistently, paralleled those for calcium, a finding which is in accord with the results of many workers (8, 11–14) in lactation experiments.

TABLE IV.

Per Cent Utilization of Calcium and Phosphorus of Food by Women in Different Weeks of Two Successive Lactation Cycles.

	Subject VI.			Subject VII.			Subject VIII.	
Lactation Cycle	A	B		A	B		B	
Wk. of lactation	60	7	27	50	7	26	7	27
Calcium.								
Breast milk	19.4	44.0	38.2	7.2	21.0	21.4	13.1	11.9
Urine	6.6	2.7	11.8	8.2	2.1	1.8	3.7	2.8
Feces	67.1	68.3	113.5	72.2	78.5	93.4	120.7	126.9
Retention	+6.9	−14.9	−63.5	+12.4	−1.6	−16.6	−37.5	−41.6
Phosphorus.								
Breast milk	7.7	16.3	11.1	4.6	15.0	10.6	8.1	7.2
Urine	49.6	61.0	77.3	41.1	43.2	24.4	56.1	14.6
Feces	31.0	26.4	34.2	36.0	47.7	46.4	36.6	39.4
Retention	+11.7	−3.7	−22.6	+18.3	−5.9	+18.7	−0.8	+38.8

"Assimilation" in this report means the difference between the total intake and the sum of the urinary and fecal excretion. It is expressed in terms of gm. and the percentage of the total intake in Table III. The body reserves were heavily taxed during early lactation in Subject VIII and during mid-lactation in Subjects VI and VIII, primarily because of increased calcium excretion in the feces in addition to the outgo in milk.

Hart and his associates (19) found a similar waste of calcium by fecal excretion, often equal in amount to the calcium of the intake. Forbes *et al.* (8) record the intake of 175 gm. of calcium oxide by a

cow in liberal milk production and a utilization of only 40 gm. of the oxide in the secretion itself; yet a negative balance persisted.

The percentage utilization of the calcium intake in breast milk, urine, feces, and retention is shown in Table IV. The increased proportion of calcium elaborated in the breast milk during high production periods, at the 7th and 27th weeks of lactation, together with the increased fecal excretion with subsequent increased loss of body reserves, is striking when compared with the results for the late stage of lactation.

The percentage distribution of calcium among the different paths of outgo (Table V) shows that the variations in the three

TABLE V.

Per Cent Distribution in Outgo of Calcium and Phosphorus from Women during Different Weeks of Two Successive Lactation Cycles.

	Subject VI.			Subject VII.			Subject VIII.	
Lactation Cycle	A	B		A	B		B	
Wk. of lactation	60	7	27	50	7	26	7	27
Calcium.								
Breast milk	20.9	38.3	23.4	8.3	20.7	18.4	9.5	8.4
Urine	7.1	2.3	7.2	9.3	2.1	1.6	2.7	2.0
Feces	72.0	59.4	69.4	82.4	77.2	80.1	87.8	89.6
Phosphorus.								
Breast milk	8.7	15.7	9.1	5.6	14.1	13.0	8.0	11.7
Urine	56.2	58.8	63.0	50.4	40.8	30.0	55.1	23.9
Feces	35.2	25.5	27.9	44.0	45.1	57.0	36.3	64.4

individuals were from 8.3 to 38.3 in breast milk, from 59.4 to 89.6 in the feces, and from 1.6 to 9.3 in urine. The lesser values for calcium in urine are found during high milk production and the greater ones during decreased secretion. Both trends of retention and distribution are in accord with the findings of Hunt and his associates (12), who studied goats at three stages of lactation. One goat was producing approximately the same amount of milk as Subject VIII.

The percentage utilization of the intake (Table IV) shows that in the cases of storage in the mid-lactation period there was a decrease in proportion in urinary excretion. The distribution of

outgo in Table V is in close agreement with the findings of other investigators (8, 11, 12, 20).

Table VI presents the ratio of calcium to phosphorus in the intake and the constituent parts of the outgo. It is interesting to note the increase in all cases in the values for the several paths of outgo from early to mid-lactation. The lowest values appear in the balances of late lactation.

The limited knowledge of the relation of calcium and phosphorus in the diets of adult human beings permits no generalizations. Turner *et al.* (20) and Meigs *et al.* (17) stated that the optimal ratio of intake for the milch cow was 1.0 to 1.5; Hart and his associates

TABLE VI.

Ca:P Ratios of Balances in Women during Two Successive Lactation Cycles.

	Subject VI.			Subject VII.			Subject VIII.	
Lactation Cycle.....	A	B		A	B		B	
Wk. of lactation.....	60	7	27	50	7	26	7	27
Food.....	0.91	0.91	0.95	0.90	1.07	1.08	1.12	1.38
Breast milk.....	2.29	2.55	3.22	1.43	1.50	2.19	1.80	2.28
Urine.....	0.12	0.04	0.14	0.18	0.05	0.08	0.07	0.27
Feces.....	1.97	2.44	3.12	1.82	1.76	2.17	3.68	4.42
Outgo.....	0.96	1.05	1.25	0.97	1.03	1.55	1.52	3.18
Retained.....	0.53	−2.14	−2.74	0.61	−0.29	*	−52.2	*

* Positive phosphorus and negative calcium retention.

(18) lay more stress upon sufficient intake of both and provision of the antirachitic factor than upon definite ratios in the food.

Roentgenograms[3] of the teeth and complete oral examinations were made simultaneously with the metabolic balance studies of lactation, but with the methods and technique used no demonstrable changes could be shown to be caused by lactation. No new cavities appeared during the first 6 months of lactation Cycle B.

The prevalence of negative calcium and phosphorus balances in animals and man has stimulated an inquiry as to whether or not, in liberal milk secretion, the body reserves are more readily avail-

[3] Samuel J. Lewis, D.D.S., kindly made the mouth examinations and roentgenograms of the teeth at the end of lactation Cycle A, and the personal dentists of the women followed the oral conditions thereafter.

able than the minerals of the food intake are. Forbes *et al.* (8) considered negative calcium balances an uncertain index of insufficiency of intake. They summarized their opinion in the following manner: "This loss appears rather to be the resultant of several factors, among which are the degree of the impulse to secrete milk, the power of the cow to digest, absorb, and assimilate calcium, and the ability of the cow to draw upon her mineral reserves." In a previous publication on the calcium and phosphorus metabolism of women during gestation (1), some of the untoward influences upon digestion, assimilation, and utilization of calcium have been considered. Bauer, Aub, and Albright (36) have shown that:

> "Bone trabeculæ have as their function not only to act as a support against the tension of muscles . . . but to serve as the most readily available calcium supply in time of need. They can be influenced by many factors. Thus prolonged administration of parathormone in rabbits will decrease their number. Likewise a prolonged negative calcium balance will diminish these same bony spicules while a high calcium diet increases the amount of cancellous bone. Regardless of whether an animal is kept on a low or high calcium diet for a long period of time, no gross changes are noted in the cortex. The trabeculæ seemingly serve as the labile supply of calcium and the cortex as the stable supply."

The superiority of the diets of these lactating mothers is demonstrated by the fact that they were taking daily between 3700 and 4700 calories, between 27.8 and 29.4 gm. of nitrogen, between 2.8 and 4.4 gm. of calcium, and between 2.9 and 4.3 gm. of phosphorus. An unusually large amount of the food elements was derived from milk, which contains the food elements in a readily available form (37) and on account of its buffer action (base minus chloride) may have an effect upon calcium and phosphorus retention (38). The lactose content may also play a part in the assimilation (39, 40). The diets also contained liberal quantities of fresh fruits and vegetables (41). In spite of a liberal intake of minerals from readily available sources, negative balances persisted, thus indicating that there are other factors influencing metabolism.

It has become apparent from the accumulated evidence in the literature, as reviewed in this and a preceding paper (1), that the acid-base properties of the food ingested, as well as the metabolic

products resulting therefrom, have a direct effect upon the calcium balance. The diets of all three subjects had a tendency to be alkaline, with an excess of approximately 50 cc. of 0.1 N base per day. This is in accord with the suggestion made for the reproductive cycle by Shohl (42), who states that "a base retention during pregnancy and lactation is the result of two causes: milk secretion causes a negative base balance, and the alkali requirement of the fetus causes a positive base balance."

It is not known to what extent calcium and phosphorus metabolism is affected during lactation by the glands of internal secretion. The basal metabolic rates[4] of the three subjects at the 27th week of lactation were as follows: Subject VI +7 per cent, Subject VII − 3 per cent, and Subject VIII +14.5 per cent.

Since there is a possible seasonal effect upon calcium utilization, it is significant to know that all balance studies were made in the fall and winter at comparable stages of lactation. It is assumed, then, that sunshine was not a determinate factor in the assimilation of calcium and phosphorus in this study.

Meigs and his associates (17) attributed the variations of calcium assimilation in cows partially to the nervous factor introduced by experimental procedure. In the present studies on the human subject, for which the same worker was maintained and the same methods of management followed during $2\frac{1}{2}$ years of special psychological, dietary, and metabolic observations, this factor, according to the subjects themselves, played a very small part. Studies on the psychological factors in human milk secretion will be reported later from this laboratory.

Charts I and II show the physiological histories of the three women during a complete reproductive cycle. They indicate a larger consumption of these elements during lactation than during pregnancy, but in early and mid-lactation there is an excessive fecal excretion of calcium resulting in negative balances. These findings demonstrate the inability of the organism to utilize the increased available food during lactation.

Attention has been called to the many complicating factors that may influence the digestion, absorption, assimilation, and excretion of calcium and phosphorus. There is little definite knowledge

[4] Grateful acknowledgment is due W. L. Brosius, M.D., for making the basal metabolism determinations.

concerning the intricate processes involved in these various phases of calcium and phosphorus metabolism in man; still less is there any adequate knowledge of the variations of these processes in the individual. Knowledge regarding the physiological changes brought about in the maternal organism during the reproductive cycle is fragmentary, and even less is known regarding the effects of frequent sexual activity. The occurrence in these subjects of frequent pregnancies followed by long and abundant milk flow

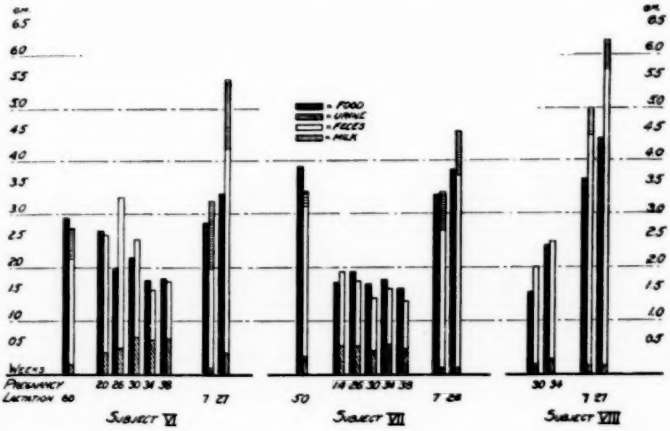

CHART I. Daily calcium exchange in pregnancy and lactation. The solid black columns illustrate the total number of gm. of calcium in the daily food intake; the plain, cross-shaded, and diagonal-shaded columns show the total outgo in calcium in the feces, breast milk, and urine, respectively. The difference between the height of the intake and outgo columns represents the calcium balance. When the solid column exceeds the outgo, calcium is being stored in the body; on the other hand, when the opposite condition exists, calcium is being lost.

makes the interpretation of the results reported herein very difficult.

It is possible that continuous calcium and phosphorus exhaustion may tend to have an overstraining effect upon the absorptive and assimilative functions. There was little opportunity for the rebuilding of the stores of these minerals within the maternal body between the reproductive cycles. It has been pointed out also

that the acid-base equilibrium of the body is affected not only by the kinds of foods eaten but also by the amount of catabolism and anabolism going on within the body itself, thus influencing the utilization of calcium and phosphorus. Subjects VI and VII experienced a loss of body weight during lactation Cycles A and B, while Subject VIII gained. Large amounts of calcium and phosphorus were continuously available in the voluntary diets chosen by the three women during their entire reproductive period; *i.e.*, in pregnancy and lactation. Either, then, there was not a sufficient

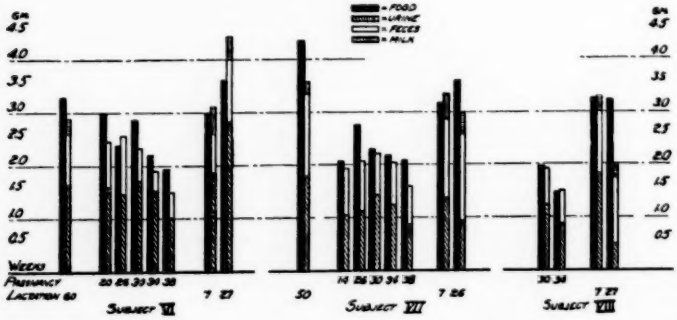

CHART II. Daily phosphorus exchange in pregnancy and lactation. The solid black columns illustrate the total number of gm. of phosphorus in the daily food intake; the plain, cross-shaded, and diagonal-shaded columns show the total outgo in phosphorus in the feces, breast milk, and urine, respectively. The difference between the height of the intake and outgo columns represents the phosphorus balance. When the solid column exceeds the outgo, phosphorus is being stored in the body; on the other hand, when the opposite condition exists, phosphorus is being lost.

amount of the calcium fixation substances present in the diet or environment, or there were other regulatory factors operating within the maternal body to inhibit the absorption and utilization of the calcium and phosphorus available in the digestive tract during the 7th and 27th weeks of lactation Cycle B. In lactation Cycle A, Subjects VI and VII were in positive calcium and phosphorus balance during the 14th and 11th months respectively.

It is essential that the interpretation of these short period data on the calcium and phosphorus metabolism in early, mid-, and late

lactation should be considered in relation to the woman's determinate individual physiological characteristics and reproductive history, and should not be generalized.

SUMMARY.

Balance studies were made on three women with superior milk production at early, mid-, and late lactation to determine the rôle of lactation in the calcium and phosphorus metabolism. The studies were conducted in the homes while the subjects consumed their usual diets and carried on their accustomed duties.

The amount of milk, together with the calcium and phosphorus elaborated in 24 hour periods at monthly intervals, was observed for two successive lactation cycles in two women and for one cycle in the third.

Negative calcium and phosphorus balances were found in early lactation in spite of large intakes of these elements.

After 6 months of continued high milk production, appreciable increases in the loss of calcium were observed. Fecal excretion played a prominent rôle in the depletion, the amount in the feces at times exceeding the total intake. Phosphorus results were variable.

In late lactation, when less milk was secreted, both calcium and phosphorus were stored in the maternal body.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE.

III. CALCIUM, PHOSPHORUS, AND NITROGEN UTILIZATION IN LACTATION BEFORE AND AFTER SUPPLEMENTING THE USUAL HOME DIETS WITH COD LIVER OIL AND YEAST.*

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It has been shown that women who had experienced long physiological demands from frequent pregnancies and prolonged abundant milk flow were in negative calcium and phosphorus balances at the 6th and 27th weeks of lactation. Although consuming diets superabundant in food constituents, with 3 to 4 gm. each of calcium and phosphorus, 27.8 to 29.4 gm. of nitrogen, and 3700 to 4700 calories per day, they had progressively lost calcium and phosphorus in appreciable excess of their intakes during the first 6 months after parturition, according to 4 day metabolic studies. Frequent metabolic studies over a period of time have made it increasingly evident that during the reproductive cycle many factors, both negative and positive, operate to inhibit the optimal utilization of calcium and phosphorus in the maternal organism. The protection of the maternal organism during

* A report will appear soon on the vitamin content of the milk of individual mothers before and after the administration of cod liver oil and yeast.

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this period of augmented physiological activity demands better methods of bringing about adequate digestion, absorption, and utilization of all food constituents. The present paper reports the results of an attempt to further these metabolic processes by supplementing the usual home diets of lactating women with cod liver oil and yeast, which have been demonstrated to have a favorable influence upon mineral metabolism.

The many complicating factors acting in diverse ways make it difficult to evaluate the significance of the results in metabolic balance studies. Some of these influences and conditions have been reviewed in the preceding papers (1, 2), and accordingly only the results that seem pertinent to this report are discussed here.

Hart, Steenbock, and Hoppert (3) demonstrated that cod liver oil increased the assimilation of calcium, thus changing negative calcium balances in milking and dry goats to positive retentions. Harvey (4) found that the administration of 10 cc. of cod liver oil daily improved the calcium utilization of the goat by reducing the fecal excretion of calcium. The effects on phosphorus metabolism were variable. Hart, Steenbock, Kletzien, and Scott (5) in an extensive series of experiments on goats found that negative calcium and phosphorus balances resulted when the antiricketic factor was not added to the basal diet. Indeed, when the non-saponifiable fraction equivalent to 80 cc. of cod liver oil was mixed with dextrin and given in capsules, negative balances persisted. Not until a comparable amount of the non-saponifiable fraction was dissolved in 15 cc. of purified corn oil prior to administration was a positive balance obtained. From their results the authors conclude that "the antirachitic factor of cod liver oil is as effective an agent in promoting calcium assimilation in a lactating animal as it is in a growing one." From these studies on goats it is evident that the antiricketic factor in cod liver oil is the substance that influences calcium assimilation, since vitamin A is removed through the saponification process. This is further proved by the results of experiments on the effects of direct irradiation of the goat (6, 7) and its food (8) with ultra-violet light. These results are similar to those obtained by Orr, Holt, and Boone (9) on growing children, by Shohl and coworkers (10, 11) on rats and dogs, and by Daniels, Stearns, and Hutton (12) on infants.

On the other hand, there seems to be a variation in the response of cows to analogous treatment. Meigs and associates (13) found no improvement in calcium and phosphorus balances when cod liver oil was administered to cows. These animals, however, were unaccustomed to taking oil over a relatively long period of time, and consequently their appetites were impaired and their food consumption decreased. More recently, Hart, Steenbock, Teut, and Humphrey (14) have demonstrated that: "Feeding

½ pound daily of cod liver oil, potent in vitamin D, showed no favorable influence upon the calcium assimilation of liberally milking cows. Vitamin D in the cod liver oil was poorly, if at all, absorbed from the intestinal tract." The direct administration of ultra-violet light, natural or artificial, has no apparent influence upon the metabolism of the cow (15, 16). Reviewing the results of studies at the Beltsville and Wisconsin laboratories on the effects of different types of food on the calcium and phosphorus metabolism of dairy cows, Meigs *et al.* (13) state that "calcium is decidedly better assimilated from fresh green material than from the same material after it has been dried and made into hay. The manner in which hay is cured makes a great difference in the rate at which calcium is assimilated from it." The milch cow evidently responds somewhat differently than the goat, dog, and infant to the antiricketic factor as found in cod liver oil and ultra-violet light.

Bogert and Trail (17) found that adding butter fat, a rich source of vitamin A, to the diet of women favorably influenced their calcium balance, and that the administration of yeast had a similar effect. Funk and Levy (18) demonstrated that vitamin B tended to increase the utilization of calcium, phosphorus, and nitrogen in the rat.

From this review it would seem that cod liver oil and yeast, containing vitamins A, B, and D, would tend to promote a more economical utilization of calcium, phosphorus, and nitrogen during the increased metabolic activity accompanying the reproductive cycle of women. Furthermore, the availability of these substances would make them of practical use in supplementing the usual home diets of nursing mothers in such a way as to stimulate the better utilization of the essential food constituents.

EXPERIMENTAL.

Procedure.

The subjects of the present investigation were found to be in negative calcium and phosphorus balances at the 7th week of lactation, with a progressively greater negativity at the 27th week (2). Subjects VI, VII, and VIII were in their 4th, 3rd, and 2nd lactation cycles, respectively, each reproductive cycle having followed closely upon the preceding one, with subsequent abundant and prolonged secretion of milk. The women, then, had been under constant biological demand for a considerable period of time.

The accustomed home diets of all three women were known to contain liberal amounts of calcium, phosphorus, and nitrogen.

Beginning March 1, 1929, supplementary additions of three 5 gm. gelatin capsules of cod liver oil and 10 gm. of yeast were superimposed daily upon the usual dietaries. Subjects VI, VII, and VIII were then in their 29th, 27th, and 33rd weeks of lactation, respectively. After the women had had these accessory food factors for 2 months, the metabolic balances were again determined. The milk production for all three women remained within the limits of daily variation throughout this interval. The daily supplements were added to the diets and are therefore included in the analysis of the food.

As is the custom in these studies of women during the reproductive cycle, the materials for the metabolic balance determinations were collected in the homes over a 4 day period. The methods of procedure followed in making the collections and chemical determinations are fully described in the preceding papers (1, 2) and are therefore omitted here. The usual metabolic collections were made and accepted macrochemical methods were used in all analyses.

Results.

Subject VI.—In the preexperimental period, at the 27th week of lactation, Subject VI showed a rapidly increasing loss of calcium reserves, even in excess of the losses at the 7th week, though the usual home diet contained an apparent superabundance of all food constituents. After cod liver oil and yeast had been administered for 2 months, and the milk production was approximately the same, metabolic balance determinations showed a restoration of both calcium and phosphorus to the body (Table I). Noteworthy retentions were observed in the experimental period: the calcium had diverged from a markedly negative balance of −2.15 gm. daily to a positive one of +0.42 gm.; the phosphorus balance progressed from −0.82 gm. daily in the preexperimental period to +1.42 gm. in the experimental period. The nitrogen retention, however, decreased slightly (from +0.72 to +0.42 gm. daily) between the two periods. The improved metabolic balances in the experimental period become increasingly significant when the constancy of the following factors is considered: the volume output of milk had maintained its usual level, within the limits of daily variations; the excretion of calcium, phosphorus, and

nitrogen in the breast milk remained practically the same; and, further, the food intake was the same. There was a striking decrease in the number of gm. of calcium and phosphorus excreted in the feces during the experimental period. These findings are in

TABLE I.

Daily Calcium, Phosphorus, and Nitrogen Balances before and after Supplementing the Usual Diet with Cod Liver Oil and Yeast.

Element.	Period.	Milk secreted.	Total intake.	Outgo.				Balance.
				Breast milk.	Urine.	Feces.	Total.	
		Subject VI.						
		cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Calcium.	Preexperimental.	3455	3.39	1.29	0.40	3.85	5.54	−2.15
	Experimental.	3365	3.41	1.17	0.16	1.66	2.99	+0.42
Phosphorus.	Preexperimental.	3455	3.61	0.40	2.79	1.23	4.43	−0.82
	Experimental.	3365	3.62	0.37	1.35	0.48	2.20	+1.42
Nitrogen.	Preexperimental.	3455	27.63	5.41	19.18	2.33	26.92	+0.71
	Experimental.	3365	27.91	5.34	19.92	2.23	27.49	+0.42
		Subject VII.						
Calcium.	Preexperimental.	2460	3.83	0.84	0.07	3.67	4.58	−0.75
	Experimental.	2490	2.76	0.83	0.13	2.18	3.14	−0.38
Phosphorus.	Preexperimental.	2460	3.58	0.38	0.88	1.69	2.95	+0.63
	Experimental.	2490	3.31	0.38	0.95	1.18	2.51	+0.80
Nitrogen.	Preexperimental.	2460	25.14	4.02	17.57	2.68	24.27	+0.87
	Experimental.	2490	25.92	4.01	17.64	2.82	24.47	+1.45
		Subject VIII.						
Calcium.	Preexperimental.	1654	4.42	0.53	0.12	5.61	6.26	−1.84
	Experimental.	1496	3.10	0.45	0.15	3.05	3.65	−0.55
Phosphorus.	Preexperimental.	1654	3.22	0.23	0.47	1.27	1.97	+1.25
	Experimental.	1496	3.62	0.19	1.37	0.87	2.42	+1.20
Nitrogen.	Preexperimental.	1654	21.79	3.13	18.57	2.40	24.10	−2.31
	Experimental.	1496	27.47	2.84	19.57	2.17	24.58	+2.89

accord with those of other investigators, notably Hart *et al.* (3, 5) and Harvey (4) who studied lactating goats, Shohl and Bennett (11) who studied dogs, and Daniels *et al.* (12) who observed infants.

Table II illustrates the distribution of the elements in the paths of outgo (milk, urine, and feces), computed in terms of total intake and total excretion. Better calcium and phosphorus utilization is evidenced by a decrease from 113.5 to 48.8 in the percentage of

TABLE II.

Per Cent of Calcium, Phosphorus, and Nitrogen Intake and Outgo in Milk, Urine, and Feces.

Element.	Period.	Total intake.				Total outgo.		
		Retention.	Milk.	Urine.	Feces.	Milk.	Urine.	Feces.
Subject VI.								
Calcium.	Preexperimental.	−63.5	38.2	11.8	113.5	23.4	7.2	69.4
	Experimental.	+12.2	34.2	4.8	48.8	38.9	5.5	55.6
Phosphorus.	Preexperimental.	−22.6	11.1	77.3	34.2	9.1	63.0	27.9
	Experimental.	+39.2	10.2	37.4	13.2	16.8	61.3	21.8
Nitrogen.	Preexperimental.	+2.6	19.6	69.4	8.5	20.1	71.2	8.7
	Experimental.	+1.5	19.1	71.4	8.0	19.3	72.4	8.0
Subject VII.								
Calcium.	Preexperimental.	−16.6	21.4	1.8	93.4	18.4	1.6	80.1
	Experimental.	−13.6	30.1	4.7	78.8	26.5	4.2	69.3
Phosphorus.	Preexperimental.	+18.7	10.6	24.4	46.4	13.0	30.0	57.0
	Experimental.	+24.3	11.6	28.9	35.3	15.3	38.0	46.7
Nitrogen.	Preexperimental.	+4.6	15.8	69.1	10.6	16.6	72.4	11.1
	Experimental.	+5.6	15.5	68.1	10.9	16.3	72.1	11.5
Subject VIII.								
Calcium.	Preexperimental.	−41.6	11.9	2.8	126.9	8.4	2.0	89.6
	Experimental.	−17.9	14.5	4.9	98.5	12.3	4.1	83.6
Phosphorus.	Preexperimental.	+38.8	7.2	14.6	39.4	11.7	23.9	64.4
	Experimental.	+33.1	5.2	37.7	24.0	7.7	56.4	35.9
Nitrogen.	Preexperimental.	−10.6	14.4	85.2	11.0	13.0	77.1	10.0
	Experimental.	+10.5	10.3	71.2	7.9	11.6	79.6	8.8

ingested calcium excreted in the feces, and from 34.2 to 13.2 in the percentage of phosphorus excreted. The percentages of total outgo show a similar reduction in the fecal excretion of calcium and phosphorus (calcium from 69.4 to 55.6 per cent, phosphorus

from 27.9 to 21.8 per cent). There was also a decrease in the urinary excretion of calcium and phosphorus.

Subject VII.—In the preexperimental period, at the 27th week of lactation, the metabolic balance determination showed that Subject VII was losing calcium at a greater rate than at the 7th week post partum. On the other hand, the phosphorus balance had progressed from a negative to a positive balance in the same interval of time. After cod liver oil and yeast had been administered prior to and during the experimental period, more favorable calcium, phosphorus, and nitrogen balances were obtained (Table I). The daily metabolic balances had changed from −0.75 to −0.38 gm. of calcium, from +0.63 to +0.80 gm. of phosphorus, and from +0.87 to +1.45 gm. of nitrogen. It is interesting to note that during the experimental period there had been a voluntarily decreased food consumption, the calcium intake having been cut from 3.83 to 2.76 gm., while the phosphorus and nitrogen remained approximately the same. The volume output of milk was slightly greater than it was during the preexperimental period, although the total secretion of calcium, phosphorus, and nitrogen in the two periods was the same.

Table II shows the percentages of total intake and total outgo of calcium and phosphorus in milk, urine, and feces, before and after the administration of cod liver oil and yeast. The percentage of ingested calcium excreted in the feces decreased from 93.4 to 78.8, and the percentage of phosphorus from 46.4 to 35.3. The percentages of total outgo show a similar reduction in the fecal excretion of calcium and phosphorus (calcium from 80.1 to 69.3 per cent, phosphorus from 57.0 to 46.7 per cent). At the same time, the percentage of ingested calcium excreted in the urine increased from 1.8 to 4.7, and the percentage of phosphorus from 24.4 to 28.9. The percentages of total outgo show a similar increase in the urinary excretion of these elements.

Subject VIII.—Like Subjects VI and VII, Subject VIII showed a greater loss of calcium at the 27th week of lactation than at the 7th. The phosphorus balance had changed from negative to positive over the same period of time (Table I). After the daily consumption of cod liver oil and yeast before and during the experimental period, there was a marked improvement in the calcium and nitrogen balances (calcium from −1.84 to −0.55

gm., nitrogen from −2.31 to +2.89 gm.), but practically no change in the phosphorus balance (+1.25 to +1.20 gm.). There was a slight decrease in the amount of milk secreted (from 1654 to 1496 cc.) with a consequent decrease in calcium, phosphorus, and nitrogen in the breast milk. The daily voluntary calcium intake

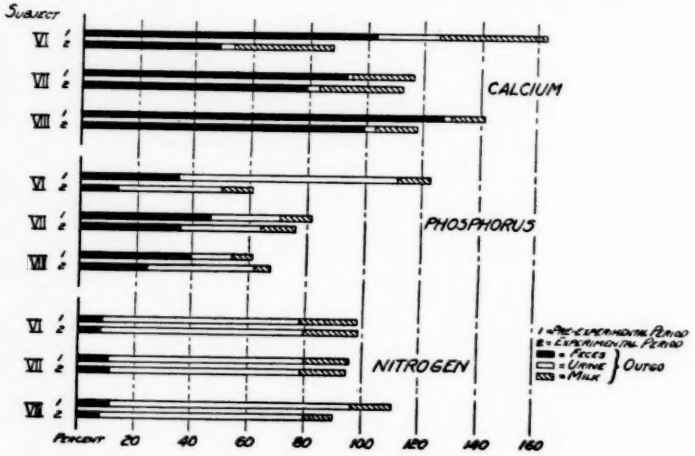

Chart I.—Percentage of ingested calcium, phosphorus, and nitrogen found in the feces, urine, and breast milk of three women before and after the administration of cod liver oil and yeast. The solid column represents the per cent of the elements excreted in the feces. There was a decreased elimination of calcium and phosphorus in all three cases during the cod liver oil and yeast period. The nitrogen balances were not appreciably affected by these supplementary foods. The plain columns illustrate the percentage of elements excreted in the urine; these results were variable. The shaded columns show the output of calcium, phosphorus, and nitrogen in the breast milk; the actual amounts of output of these elements fell within the range of the daily fluctuations in milk production for these subjects, not only during their preexperimental and experimental periods but during a 6 months period.

decreased in the experimental period from 4.42 to 3.10 gm., while the phosphorus increased from 3.22 to 3.62 gm., and the nitrogen from 21.79 to 27.47 gm.

Table II shows the percentages of total intake and total outgo of calcium and phosphorus in milk, urine, and feces, before and

after the administration of cod liver oil and yeast. There was a notable decrease in the fecal excretion of all elements, but, with the exception of nitrogen, the percentage of the elements excreted in the urine increased. The percentage of fecal calcium decreased from 126.9 to 98.5, the percentage of fecal phosphorus from 39.4 to 24.0, and the percentage of fecal nitrogen from 11.0 to 7.9. The percentage of urinary nitrogen decreased from 85.2 to 71.2, but the percentage of urinary calcium increased from 2.8 to 4.9, and the percentage of urinary phosphorus from 14.6 to 37.7. In terms of total outgo, there was a decrease in the elimination of all three elements in the feces, and, in the urine, a decrease in nitrogen and an increase in calcium and phosphorus.

TABLE III.

Calcium, Phosphorus, and Nitrogen Retention before and after Supplementing Home Diets with Cod Liver Oil and Yeast.

The results are expressed in gm. daily per kilo of body weight.

Calcium.			Phosphorus.			Nitrogen.		
Subject VI.	Subject VII.	Subject VIII.	Subject VI.	Subject VII.	Subject VIII.	Subject VI.	Subject VII.	Subject VIII.
Preexperimental period.								
−0.033	−0.012	−0.023	−0.013	+0.010	+0.016	+0.010	+0.014	−0.029
Experimental period.								
+0.007	−0.006	−0.007	+0.022	+0.013	+0.015	+0.006	+0.024	+0.035

Chart I shows the metabolic effects of supplementing the usual home diets of the three lactating women with cod liver oil and yeast. The reduction in the percentage of ingested calcium and phosphorus excreted in the feces, for all three of the women, indicates a favorable effect upon calcium and phosphorus metabolism. The nitrogen metabolism is apparently not appreciably affected by such a procedure.

Table III illustrates the calcium, phosphorus, and nitrogen retention in units of gm. daily per kilo of body weight for the three nursing mothers, prior to and during the balance period of supplementing the home diets daily with 15 gm. of cod liver oil and 10 gm. of yeast.

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DISCUSSION.

Healthy women who were producing large quantities of milk and habitually consuming from 3 to 4 gm. of calcium per day in the diet were unable to assimilate a sufficient amount of this element to satisfy the demands of milk production and at the same time maintain calcium equilibrium. The customary diets of these subjects contained quantities of milk varying from 1 to 3 quarts per day, and a liberal supply of green vegetables, fresh fruits, cheese, and eggs, thus making up a food intake that is generally considered adequate in every respect for the nursing mother. It is evident that some calcium-fixating factor is absent or there are other influences operating to inhibit calcium utilization during heavy milk production.

In this study it has been demonstrated that daily additions of 15 gm. of cod liver oil and 10 gm. of yeast to the apparently adequate diets of three nursing women appreciably improved their calcium assimilation, though positive calcium balances did not always result. This augmented utilization of calcium has been accounted for by the decreased fecal output of the element. Such findings are in accord with those of Hart and his collaborators (3, 5) and Harvey (4), who experimented with lactating goats.

Forbes (19), Miller (20), and Hunt (21) and their coworkers have found that when milk production in cows and goats decreased in late lactation there was a tendency towards storage of both calcium and phosphorus. The milk output of the three women under observation remained practically constant for 5 months preceding the preexperimental period and also during the 2 months of cod liver oil and yeast administration. The increased calcium utilization during the experimental period cannot, therefore, be attributed to a change in the volume of milk secreted.

The phosphorus utilization of the three subjects was improved by supplementing their usual diets with cod liver oil and yeast. The phosphorus balances, however, did not improve as markedly as the calcium balances did. These results are in agreement with those of Harvey (4), who found that in the goat the phosphorus results were variable and that the increased utilization of phosphorus was coincident with decreased fecal phosphorus.

The lactating woman has a double biological demand for min-

erals. A supply is required for milk production over and above the amount that is lost in the urine and feces from the usual anabolism and catabolism of tissues. It has become evident in the studies of these three subjects that not all the mineral matter in the food intake was digested and absorbed, much less a sufficient amount utilized to satisfy the demands of lactation. There seem to be a number of factors that make assimilation of certain minerals difficult, even when an abundance of them is present in the food. In reference to calcium and phosphorus metabolism, Hart *et al.* (22) and Meigs *et al.* (13) have suggested that certain irregularities may often be caused by individual differences in the state of the calcium and phosphorus stores. The women of this study had had little opportunity for sexual rest and had therefore been under continuous augmented physiological activity for a considerable period of time.

Individual differences in the metabolism of calcium and phosphorus may depend also upon individual physiological variations. During the experimental period, Subjects VI and VII lost in body weight, while Subject VIII gained.

Irregularities in the retention of calcium and phosphorus in the intestine sometimes occur. There are individual differences in the rate of emptying the intestine and in the rate at which it absorbs and excretes these mineral elements. The pH of the intestinal tract is known to influence calcium and phosphorus absorption. Increased acidity in the intestinal tract markedly increases the solubility of calcium phosphate, and so favors its utilization, while alkalinity contributes to poor calcium and phosphorus utilization (23). Bergeim (24) has demonstrated that calcium and phosphorus are absorbed from different parts of the gastrointestinal tract and that cod liver oil lowers the pH of the entire intestinal tract, thus decreasing the elimination of both calcium and phosphorus and stimulating their utilization. These findings have been verified by other investigators (25–27).

Greenwald and Gross (28) have shown that administration of cod liver oil stimulates parathyroid tissue to increased activity. These writers state that:

"If we assume that the parathyroid hormone, directly or indirectly, increases the calcium-dissolving power of the plasma and that cod liver oil stimulates the parathyroids, it may readily be seen that the addition of cod liver oil to a diet rich in calcium that is being fed to young animals, in

TABLE IV.

Percentage of Ingested Calcium and Phosphorus Found in Urine, Feces, and Milk after Supplementing Diets with Cod Liver Oil or Ultra-Violet Light.

Authors.	Subject.	Diet and experimental supplements.	Average of ingested Ca excreted.			Average of ingested P excreted.		
			Urine.	Feces.	Milk.	Urine.	Feces.	Milk.
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Orr, Holt, Boone (9).	Infants.	Cow's milk and sugar.	0.7	93.1		31.2	67.3	
		Infant irradiated.	4.1	52.8		65.0	23.3	
Daniels, Stearns, Hutton (12).	Infants.	Pasteurized feeding. No additions.	3.04	93.83		50.41	43.33	
		Pasteurized feeding. Cod liver oil prior to balance.	5.02	77.30		51.66	22.97	
		Pasteurized feeding. Cod liver oil prior to and during balance.	5.50	69.92		50.26	20.68	
		Boiled feeding. No additions.	2.90	85.08		46.92	42.35	
		Boiled feeding. Cod liver oil prior to balance.	4.68	75.96		51.91	26.20	
		Boiled feeding. Cod liver oil prior to and during balance.	4.54	67.13		52.93	22.87	
		Irradiated olive oil.	6.93	56.92		54.83	13.09	
		" milk.	4.47	64.20		57.17	22.21	
	Ricketic child.		1.72	100.11		22.48	72.19	
Shohl, Bennett, Weed	Rats							

	Ricketic child.								
Shohl, Bennett, Weed (10).	Rats.	Steenbock ricketic diet.	17	57		4	69		
		2 per cent cod liver oil.	10	55		3	60		
		Irradiated corn + 2 per cent cod liver oil.	12	53		2	49		
Hart, Steenbock, Hoppert (3).	Goats.	Grain, casein, oat straw.	20.0	87.0	0.5				
		Cod liver oil.	25.2	74.3	0.4				
Hart, Steenbock, Kletzien, Scott (5).	Goats.	Alfalfa hay, corn, wheat bran, oil meal.	0.6	131.7	18.4				
		Non-saponifiable fraction of cod liver oil in corn oil.	1.4	85.9	14.6				
Henderson and Magee (7).	Goats.	Hay and concentrates.	2.4	83.5	12.5	0.64	74.21	24.64	
		Animal irradiated.	2.5	78.1	11.4	0.55	71.96	23.48	
Subject VI.	Woman.	Ordinary home diet abundant in Ca and P.	11.8	113.5	38.2	77.3	34.2	11.1	
		Cod liver oil and yeast supplements.	4.8	48.8	34.2	37.4	13.2	10.2	
" VII.	Woman.	Same.	1.8	93.4	21.4	24.4	46.4	10.6	
			4.7	78.8	30.1	28.9	35.3	11.6	
" VIII.	Woman.	Same.	2.8	126.9	11.9	14.6	39.4	7.2	
			4.9	98.5	14.5	37.7	24.0	5.2	

which calcium deposition is rapid, or to lactating animals, in which also large amounts of calcium are removed from the blood, might lead to an increased absorption of calcium; whereas the same addition, particularly when combined with a calcium-deficient diet, in the case of adult and non-lactating animals, in which there is no biological imperative demand for calcium, would lead to an increased excretion of calcium."

The literature on the effect upon young and growing animals of the antiricketic factor, in the form of ultra-violet light and cod liver oil, demonstrates that, as in certain lactating animals, these supplements promote calcium assimilation in the young rat (10, 24–26), in the rabbit (29), in dogs (11), and in infants (12). Table IV gives a summary of some of the literature on the percentage of ingested calcium and phosphorus found in the urine, feces, and in some cases, milk, after supplementing diets with cod liver oil or irradiating with ultra-violet light. It is evident that the increased absorption of these minerals during the period of antiricketic treatment is primarily due to a reduction in the fecal excretion of the minerals.

Bogert and Trail (17), in studying the influence of yeast upon calcium assimilation in four normal women, found that there was a lowered excretion of calcium in three of the subjects, owing to the excretion of a smaller percentage of calcium in the feces and to decided changes in the calcium balance. The negative balance was changed to positive in two cases, while the third subject was brought practically into equilibrium in this period. The ingestion of yeast had little effect upon the calcium excretion of the fourth subject. These results are in accord with those of Funk and Levy (18) who found that adding yeast to the diet of rats improved their mineral balances.

Yeast, which is a rich source of the vitamin B complex, has been alleged to have a stimulating effect upon the appetite. It should be noted that Subject VI in this investigation consumed the same amount of food in the experimental period as she did in the pre-experimental period, while Subjects VII and VIII voluntarily decreased their food consumption. It is apparent, then, that in these observations appetite was not a factor in furthering calcium retention. The daily yeast supplements were included in the total calcium and phosphorus intake of the women.

The results reported in this paper obviously have a direct bearing upon the physiological processes of the maternal organ-

ism during the child-bearing and child-rearing stages. They also point directly to a great need for further information concerning the calcium and phosphorus metabolism of the average nursing mother. For the mutual good of the mother and her infant, it is important to know to what extent the mother is able to give up her stores of calcium and phosphorus during the reproductive cycle without harm to the child or herself. It would be helpful, also, to know what symptoms are produced in the maternal body by the various stages of mineral depletion. Of still greater importance is the finding of a simple, economical, and effective method of stimulating a better utilization of all the essential food constituents in the customary diets of nursing mothers.

SUMMARY.

1. Calcium, phosphorus, and nitrogen balances were determined on three nursing mothers before and after supplementing their customary home diets with cod liver oil and yeast.

2. Daily feedings of 15 gm. of cod liver oil and 10 gm. of yeast over a period of 2 months when the milk flow persisted at its highest ebb stimulated better calcium and phosphorus utilization in all three cases.

3. During the experimental period the calcium assimilation was greatly improved. In Subject VI a marked negative balance was converted into a positive one, and in Subjects VII and VIII there was an appreciable reduction of the elimination of calcium.

4. The phosphorus utilization was also favorably affected; Subjects VI and VII, who were in positive phosphorus balance in the preexperimental period, showed an even greater retention after 2 months on cod liver oil and yeast; Subject VIII experienced a progression from a negative to a positive phosphorus balance.

5. The decrease of fecal excretion of both calcium and phosphorus, absolutely and relatively, during the experimental period illustrates a marked alteration in metabolism and accounts for a greater assimilation of these elements.

6. The nitrogen metabolism was inappreciably altered during the interval of observation.

7. These three lactating women experienced a more economical utilization of their available food constituents and a greater feeling of well being after the addition of cod liver oil and yeast to their home diets.

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STUDIES ON THE INACTIVATION OF CATALASE.*

II. INACTIVATION BY ULTRA-VIOLET RADIATION AT DIFFERENT HYDROGEN ION CONCENTRATIONS.

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(Received for publication, December 11, 1929.)

In a previous paper (1) the inactivation of catalase by heat at different pH was studied. It was discovered that the heat inactivation depends upon the hydrogen ion concentration of the enzyme solution which affects both the velocity and the extent of the reaction. It was also found there that 65° was the critical temperature for the enzyme preparation since at this temperature the catalase was instantly inactivated at all pH. It was further found that the degree of inactivation at different temperatures follows a rather characteristic curve, the catalase being most stable at pH 6.0 to 6.5 and losing its activity somewhat more rapidly on the acid than on the alkaline side of that pH. Catalase does not manifest a distinct optimum hydrogen ion concentration for its activity, this being covered by a range from about pH 6.5 to 8.5, so that the condition for the maximum stability of the enzyme towards heat does not exactly coincide with this range but falls only within its outside acid margin. This observation suggested that attempts to determine the chemical nature of the enzyme from its activity at different hydrogen ion concentrations must necessarily lead to erroneous conclusions and that the study of the

* The paper "Studies on the Effect of Temperature on the Catalase Reaction. VI. Heat Inactivation of Catalase at Different Hydrogen Ion Concentrations" will be considered as the first contribution on "Studies on the Inactivation of Catalase," of which the present paper is the second contribution.

resistance of the enzyme to various injurious factors at different pH would yield more reliable results for appraising its physicochemical nature. With this in view the study of the inactivation of catalase at different hydrogen ion concentrations by means of ultra-violet radiation was next undertaken.

The experiments were performed with the same catalase preparation used in the previous investigation. This was prepared from beef kidneys, the parenchymatous portion of which was finely ground and extracted several times with an equal weight of water saturated with chloroform. The combined extracts were passed through a Sharples centrifuge yielding a clear concentrated catalase solution which was freed of traces of hemoglobin and of a large part of its protein content by shaking with one-fifth its volume of chloroform. The filtered solution, preserved with a little chloroform, keeps its enzymatic activity unaltered for a very long time. A further precipitate forms on standing which may be filtered off without affecting the enzymatic strength of the solution.

The method for determining the catalase activity following various degrees of radiation with ultra-violet rays was essentially the same as employed in previous experiments. The activity was measured gasometrically by determining the volume of oxygen set free from 0.357 N hydrogen peroxide (equivalent to 100 cc. of O_2) in a reaction mixture of a total volume of 50 cc. at 21°. In order that the results should be strictly comparable in a quantitative way, the enzymatic concentration required to decompose 70 per cent of the hydrogen peroxide (yield 70 cc. of O_2 at 0° and 760 mm. of Hg) is taken as a basis. The degree of inactivation of the catalase resulting from radiation is then determined quantitatively from the amount of the weakened enzyme required to produce the same decomposition of hydrogen peroxide, the activity being expressed as the reciprocal of the enzyme concentration needed. In these experiments it was found more expedient to standardize the activity of the enzyme separately for each pH investigated. The desired pH was secured by buffering the solution with an appropriate mixture. In all but the experiments with the highest pH the Kolthoff phosphate buffers were used. The exact enzyme concentration at each pH required to give a 70 per cent decomposition of the

hydrogen peroxide is readily obtained when the results of a series of determinations with varying enzyme concentrations are plotted. This invariably gives a curve which is a straight line and the exact enzyme concentration effecting the required decomposition is easily got from the graph. An enzyme solution, a definite volume of which will set free 70 cc. of oxygen, is then prepared from the stock preparation. This solution, spread out in a thin layer in a wide dish, was exposed to the action of ultra-violet radiation. The solution was stirred during the entire time of exposure, and samples were withdrawn at regular intervals of

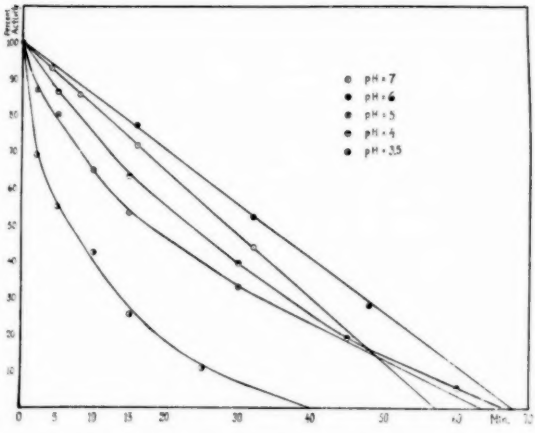

FIG. 1. Inactivation of catalase solution exposed to ultra-violet radiation at 50 cm.

radiation. The source of the radiation was a Hanovia quartz mercury vapor lamp operated at 110 volts and 5 amperes at a distance of either 25 or 50 cm.[1] Three sets of experiments were performed at each pH to determine the degree of inactivation. First a set of experiments was made to find out the concentration of the enzyme preparation necessary to yield the 70 per cent decomposition of the hydrogen peroxide at the desired pH (stand-

[1] To Dr. Carlton Pierce, Professor of Roentgenology, I am greatly indebted for placing at my disposal the quartz mercury lamp and for his cordial cooperation in standardizing the instrument.

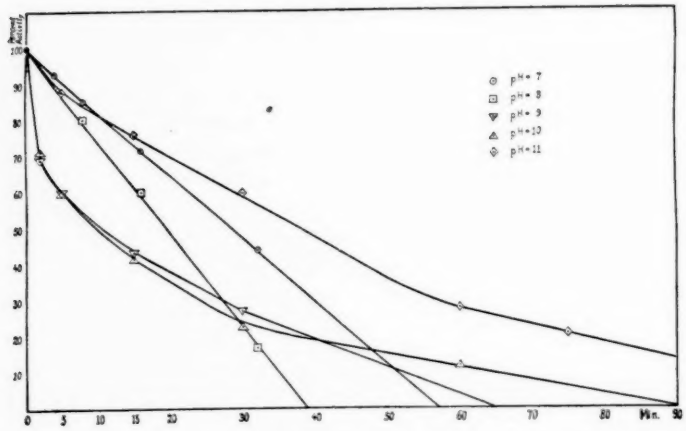

FIG. 2. Inactivation of catalase solution exposed to ultra-violet radiation at 50 cm.

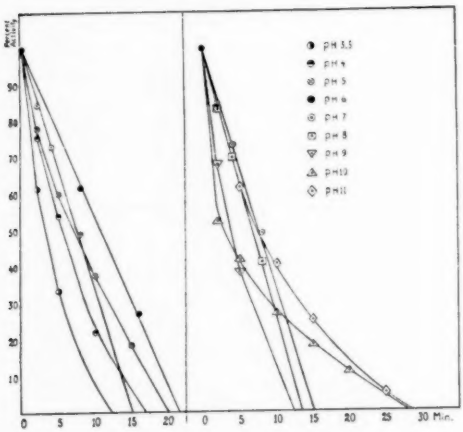

FIG. 3. Inactivation of catalase solution exposed to ultra-violet radiation at 25 cm.

ardization). A second set of experiments was made with the enzyme preparation radiated for varying lengths of time, the catalase activity being determined with the same amount of the radiated enzyme. The time required for the complete inactivation is thus determined. A third set was then performed exactly as before, samples being withdrawn at the same intervals of radiation, but the residual activity was determined on variable amounts of the enzyme solution. The longer the radiation progressed and the smaller, therefore, was the residual catalase activity, the larger was the sample employed in the experiment. By plotting the quantity of the enzyme radiated a definite length of time as abscissæ and the catalytic activity in cc. of oxygen set free as ordinates, the concentration necessary to effect the standard enzymatic work of 70 per cent decomposition is found from the graph. The zero time sample (*i.e.* the enzyme solution before being radiated) was always adjusted to give this amount of decomposition and this catalase activity was given the value of 100. The residual activity of this catalase solution at various intervals during the radiation varies inversely as the quantity of the solution required compared to that found for the zero sample.

This may be demonstrated on a specific example. At pH 4.0, 9.5×10^{-2} cc. of the stock catalase preparation were required to liberate at 21° 70 per cent of the oxygen available in the H_2O_2 used. On being exposed to ultra-violet radiation at a distance of 50 cm., progressively larger amounts of the enzyme preparation were needed to accomplish the same catalytic action. Thus:

Time of exposure. *min.*	Enzyme for 70 per cent decomposition. *cc.*	Residual activity. *per cent*
0	9.5×10^{-2}	100
5	11.0×10^{-2}	86.4
15	15.0×10^{-2}	63.3
30	24.0×10^{-2}	39.5
40	52.5×10^{-2}	18.2
60	172.5×10^{-2}	5.5

The results of the experiments are presented in a series of curves. In Fig. 1 the course of inactivation when the properly buffered catalase solution has been exposed to ultra-violet radiation at 50 cm. is shown on the acid side and in Fig. 2 on the alkaline side of

pH 7.0. Fig. 3 shows the same thing when the exposure was made at half that distance; namely, at 25 cm. At the shorter distance the inactivation, of course, is much more rapid but the course of the inactivation is practically the same. At the 25 cm. distance there is a tendency for the temperature of the radiated solution to rise but this did not exceed 8° and we can therefore be sure that the change in temperature is not a factor in the inactivation reaction, inasmuch as it has been shown before that no appreciable heat inactivation of catalase occurs below 50°.

We observe from this series of curves that at 25 or 50 cm. the inactivation of the catalase at pH 6, 7, and 8 is a linear function of the time of exposure; in other words, it proceeds at a uniform rate. However, both on the acid and alkaline side of this pH range there is no longer this direct relationship between the inactivation and time of radiation but the rate varies, being much more rapid during the first few minutes of radiation and becoming slower subsequently. In fact, at pH above 8.0 the inactivation becomes even slower than at pH 6.0 at which pH the catalase shows a maximum resistance to the ultra-violet rays, just as previously we found a maximum resistance to heat at that pH. On the alkaline side of pH 8.0 the complete inactivation is not attained in less than an hour or even a longer length of time.

At pH less than 6.0 the time required for the complete inactivation is not materially different from that at pH 6 or 7, but the course of inactivation is distinctly different, as is revealed at once by an examination of the series of curves both at the 25 and 50 cm. exposure. At pH 3.5 the inactivation already proceeds very rapidly and is completed in about two-thirds of the time required at pH 4 to 6. But at this low pH the enzyme becomes very unstable and even undergoes spontaneous inactivation. This factor necessarily complicates the experimental procedure, but both in this instance, and more particularly on the alkaline side at pH 11, where this spontaneous inactivation is even more serious, the standardization is carried out at the end of a specified length of time. All the experiments on the radiated enzyme are conducted after the same lapse of time so that the changes due to spontaneous inactivation are automatically corrected. Of course, where the spontaneous inactivation becomes so extensive as to overshadow the inactivation brought about by the

specific factor studied, such procedure could lose much of its significance and for this reason no attempts were made to study the inactivation by ultra-violet radiation at pH less than 3.5 or above 11.

At pH 6, 7, or 8, as was already mentioned, the inactivation is a linear function of the time of radiation and the rate increases from pH 6 to 8. With further increase of the pH (9 and 10) we note again, as was observed on the acid side at pH 3.5 to 5, that the inactivation proceeds at variable rates, being most rapid for the first few minutes and slowing down subsequently when the curves flatten out. The time required for complete inactivation becomes progressively greater as the pH increases. At pH 11 the situation is particularly striking, as may be seen from the corresponding curves in Figs. 2 and 3. At this pH the inactivation commences and proceeds for 5 minutes in a manner very similar to that observed at pH 8, and is therefore appreciably slower than at either pH 9 or 10. Then the rate of inactivation becomes very slow so that at the long exposure (50 cm.) the reaction is not completed, even after 75 minutes of radiation. Owing to the fact that spontaneous inactivation of the enzyme becomes a factor in experiments at such alkalinity, it is not practicable to prolong the experiments beyond that time. However, at the 25 cm. exposure complete inactivation was achieved in about 30 minutes, which is about twice the time required at pH 8.0.

The peculiar behavior of catalase at pH 11, manifesting itself in the extremely slow inactivation by ultra-violet radiation, is apparently not due to the nature of the buffer employed. The first experiments were made with a glycine-NaOH mixture as buffer for the pH 11. Since the buffer system employed in the other experiments was a phosphate mixture, we repeated these experiments, using a phosphate-NaOH mixture instead, with exactly the same results. We are justified, therefore, in concluding that the stabilizing effect and the increased resistance of the enzyme to radiation are the result of the high OH concentration of the medium.

The influence of the ultra-violet radiation at different hydrogen and hydroxyl ion concentrations is demonstrated most clearly in Fig. 4, where the residual catalase activities for a given time of exposure are plotted as ordinates against pH as abscissæ. We

note, in the first place, that at pH 6.0 the catalase is most resistant to the action of ultra-violet radiation; secondly, that at pH below 4 this resistance drops very abruptly. In this respect the inactivation by ultra-violet radiation is like that found previously for heat inactivation, and this is also manifested at the alkaline range of pH 8 to 9. With further increase in the OH ion concentration, however, and especially at an exposure of 50 cm., the inactivation requires considerably longer periods for its completion. If we tabulate the data on this point we get the following series of values:

pH	Min. required for complete inactivation. At 25 cm.	At 50 cm.
3.5	12	39
4.0	17	66
5.0	20	63
6.0	21.5	67
7.0	15	57
8.0	13	39
9.0	12 (?)	65
10.0	27	90
11.0	28	(?)

At pH 3.5 the inactivation proceeds very rapidly and is completed in about two-thirds of the time required at pH 4 to 6, while at pH above 9 the enzyme seems to regain its resistance to the ultra-violet radiation so as even to exceed that at pH 6. In other words, in an excess of OH ions the enzyme must undergo some transformation imparting to it a protection against the inactivating effect of radiant energy. The nature of this effect is not clear.

DISCUSSION AND SUMMARY.

Waentig and Steche (2), experimenting with different portions of the visible spectrum, found that the catalase activity was affected in varying degrees depending upon the purity of their enzyme preparation. The inactivation by the light, however, was such as to indicate that the ultra-violet is the most effective portion of the spectrum. These investigators found, furthermore, that the inactivation by light was greater in an alkaline than in either an acid or neutral medium. Without actual knowledge of the hydrogen ion concentration this statement is, of course,

of no particular significance since our experiments also show that at pH 8 the catalase is more affected than at pH 4 to 7, yet at greater alkalinity the enzyme shows increased resistance. Talarico (3), experimenting on catalase from horse liver, observed that red and green light had no effect, while the other parts of the spectrum inactivated the enzyme in the order blue > violet > yellow.

More recently Pincussen studying the effect of ultra-violet radiation on enzymes extended his experiments also to catalase (4).

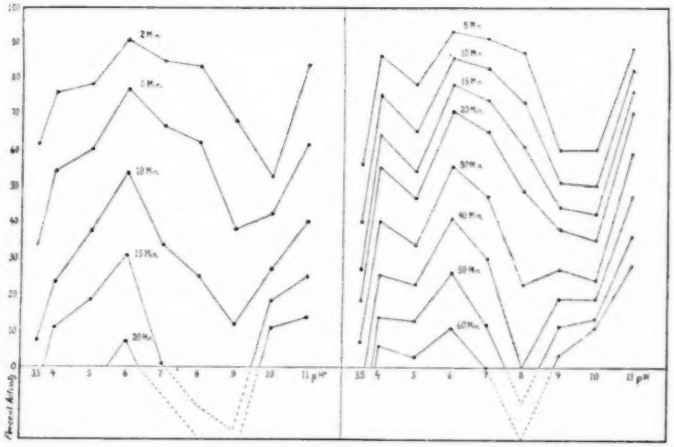

FIG. 4. Per cent of residual catalase activity following exposure to ultra-violet radiation at 25 and 50 cm. at different pH and for different lengths of time.

Using a quartz mercury vapor lamp operated at 60 volts and 2 amperes at a distance of 10 cm., he radiated catalase solutions for a period of 10 to 40 minutes. He varied the hydrogen ion concentration of the medium from pH 6.24 to 7.35 and claims to have found the greatest inactivation of the enzyme at about pH 6.8 which, he assumes, is also the optimum pH for the catalase activity. On the basis of these assumptions Pincussen finds that his results on the inactivation of catalase fit his general idea of the inactivation of enzymes by radiant energy best expressed by a

quotation from his own paper: At pH 6.8 the activity of the catalase is injured most seriously by light (?) which corresponds to our experience with other enzymes also. According to the results obtained by us so far, the pH at which inactivation through radiation is greatest may be regarded also as the pH at which the enzyme shows optimum activity. This fact gives us a method for determining the optimum hydrogen ion concentration for enzyme action.

Both the observation and the general conclusion which Pincussen draws from this are directly contradicted by our experimental findings on the radiation effect on catalase. In the experiments where the inactivation was brought about by ultra-violet radiation, as well as in the previous experiments on inactivation by heat, the catalase was least affected at pH 6.0, which in the case of catalase corresponds to the acid side of the optimum pH range for its catalytic activity. As was pointed out previously, catalase activity shows no definitely limited pH optimum. On the contrary, judged by the rate of its inactivation, catalase seems to have an optimum stability at pH 6.0 which is apparently its isoelectric point. We are unable to offer an explanation of the second peak of maximum resistance at pH 11.

Another point of interest in our experiments is the fact that the curves of inactivation of catalase at pH 6, 7, and 8 are straight lines, which is not the case at other pH. Baker and Nanavatty (5), studying quantitatively the effect of ultra-violet radiation upon bacteriophage, show that in the case of both bacteriophage and bacteria the curve of inactivation is of the type with a steep drop in the beginning followed by a more or less gradual descent subsequently. In other words, the curves are of the kind shown by our catalase preparation at pH 3.5, 4, 5, and also at pH 9 and 10. The curves of inactivation they obtained with complement, amboceptor, and various enzymes are of the type shown by our catalase at pH 6, 7, and 8. Baker and Nanavatty seem to imply from their evidence that the straight line curve of inactivation is peculiar for enzymes, whereas the other type is characteristic for living things, with the obvious implication that bacteriophage must be a living thing rather than an enzyme. This differentiation on the basis of the course of inactivation by ultra-violet radiation seems to lose its significance in the light of our experi-

ments which show that both types of curve are realized with an enzyme solution (catalase) and that the determining factor seems to be primarily the hydrogen ion concentration of the medium in which the inactivation process takes place.

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CAROTENE.

I. THE OXYGEN EQUIVALENT DETERMINED WITH POTASSIUM PERMANGANATE IN PYRIDINE SOLUTION.

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The object of the investigation of which this paper constitutes the first of a series is to describe more accurately the photosynthetic apparatus of the plant. One of the simplest leaf pigments is carotene, an unsaturated hydrocarbon, which is universally present in the chloroplasts, though its chemical structure and physiological rôle are as yet not determined.

Recently Kuhn and his collaborators (1) have determined the "Oxydationszahl" of the plant pigments, crocetin and bixin, and have employed this value to substantiate the chemical structures proposed by them for these compounds. This value which we prefer to designate as the *oxygen equivalent* was obtained from the amount of potassium permanganate reduced per mol of organic substance. The oxygen equivalent is therefore defined as the number of oxygen atoms used by 1 mol of a substance when oxidized with potassium permanganate.

Escher (2) attempted to determine the oxygen equivalent of carotene suspended in water, but found that various treatments yielded different values. His highest value was 42 atoms per mol of carotene.

Realizing the usefulness of the oxygen equivalent in theoretical considerations of chemical structure, we attempted to repeat this work. A weighed amount of carotene was sealed in a tube with a measured amount of standard alkaline potassium permanganate and heated at 105° for 7 days. But the results obtained by this method were of little value, because there was still caro-

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tene present at the end of the experiment. Furthermore, we felt that it would not be entirely reliable to estimate the excess of permanganate present after the reaction had proceeded for such a long time at that temperature, even though all the substance had been oxidized.

In order, therefore, that results might be obtained in a more satisfactory way, we decided to use a liquid which would dissolve both carotene and potassium permanganate and would react only very slowly if at all with the permanganate. Of the two solvents which seemed most promising, acetone and pyridine, the former was soon excluded because it was neither a good solvent for carotene nor was it stable in the presence of permanganate. Pyridine was found to be an excellent solvent for carotene and reasonably stable when kept with potassium permanganate at temperatures below 40°. Oxidations with permanganate in pyridine solution are not new (3, 4), but so far as we have found they have never been followed quantitatively.

Determination of Oxygen Equivalent of Carotene.

In the determination of the oxygen equivalent of carotene, the pyridine used was a C.P. grade which was purified by distillation from potassium permanganate. On refractionation the portion collected for use distilled in the range 112–115.5°. This pyridine was then saturated at room temperature with potassium permanganate and was allowed to stand for approximately 24 hours. The undissolved permanganate and any manganese dioxide formed were then removed by filtration through a Jena glass filter. The filtrate was used immediately in the reaction tubes.

The substances which were to be oxidized were dissolved in a definite volume of purified pyridine. The reaction tubes were ordinary soft glass test-tubes. These were divided into two groups, A and B. Group A was used for the oxidation of the substance and Group B for the controls. To each of the test-tubes in Group B were added 3.0 cc. of pyridine, and to each tube in Group A, 3.0 cc. of the pyridine solution containing the substance to be oxidized. Then 10.0 cc. of the standard pyridine-permanganate solution were added to each tube, first to those of Group B and then of Group A. The tubes were stoppered with cork stoppers covered with lead foil. Four of each of the groups

were then put into a constant temperature bath at 37.5° and one of each group was titrated immediately to give the initial reading.

The estimation of the quantity of permanganate reduced was made as follows: A definite volume of a standard oxalic acid solution was pipetted into 200 cc. of water containing 20 cc. of concentrated sulfuric acid, and the resulting solution heated to 55°. The contents of the reaction tubes were slowly poured into this solution with vigorous stirring. A thermometer was used as a stirring rod so that the temperature could be observed easily and controlled within narrow limits. The walls of the reaction tubes were well rinsed with the oxalic acid solution to dissolve all of the manganese dioxide adhering to them and then with distilled water. The excess oxalic acid was titrated with standard permangate solution until 1 drop gave a pink color which remained for 10 seconds at a temperature of 50° ±2°. This accurate temperature control is necessary, for if the temperature is too high the rate of reaction between pyridine and acid permanganate will be too rapid, and if the temperature is too low the oxalic acid cannot be determined accurately. Tubes A and B, removed from the constant temperature bath simultaneously, were titrated at as nearly the same time as possible. From the data obtained in this way the number of atoms of oxygen used per mol of carotene was estimated, the oxygen equivalent being equal to:

$$\frac{\text{Equivalents of } KMnO_4 \text{ used}}{\text{Mols of carotene} \times 2}$$

The experimental results and calculations are given in the appended protocol.

In order to check the method, cinnamic acid, which contains one aliphatic double bond, was used. This substance gave an oxygen equivalent of 4.97. The theoretical value on the basis of the following equation is 5.00: $C_6H_5CH{:}CH\cdot COOH + 5O \rightarrow C_6H_5COOH + 2CO_2 + H_2O$.

By the same method the oxygen equivalent of carotene was determined to be 41.97. This is in good agreement with the highest value, 42, found by Escher.

Protocol and Calculations.

The quantity of potassium permanganate which was used by the organic compound undergoing oxidation was calculated in the

following manner. Let A be the volume of oxalic acid used in each case, B the volume of permanganate necessary to oxidize the excess of oxalic acid not used by the permanganate left in the control tube, and C the volume of permanganate left in the reaction tube at the same time. The amount of permanganate used by the substance undergoing oxidation is equal to the volume of permanganate left in the control minus the amount left in the reaction tube, or

$$(A - B) - (A - C) = (C - B)$$

TABLE I.

Determination of Oxygen Equivalent of Cinnamic Acid.

The volume of oxalic acid used was 10.0 cc. of 0.1 N × 1.007 in each experiment; the weight of cinnamic acid in each reaction tube was 0.00573 gm. or 0.00003873 equivalents.

Time, *hrs.*	0.00	1.17	3.75	6.83	28.50
Back titer on reaction mixture (C), *cc.*	5.65	6.24	6.44	6.56	6.80
Back titer on control (B), *cc.*	2.42	2.54	2.64	2.73	3.06
$C - B$, *cc.*	3.23	3.70	3.80	3.83	3.74
Equivalents of $KMnO_4$	0.000323	0.00037	0.00038	0.000383	0.000374
Equivalents of $KMnO_4$ / Equivalents of cinnamic acid	8.34	9.55	9.84	9.89	9.66
Oxygen equivalent, found	4.17	4.78	4.92	4.94	4.83

From this volume the number of equivalents of oxidizing material necessary to oxidize 1 mol can be determined from the quotient,

$$\frac{\text{Equivalents of permanganate used.}}{\text{Mols of sample}}$$

To obtain the oxygen equivalents this quotient must be divided by 2, because the oxidation equivalent of oxygen is 2.

In Table I the experimental data for the oxidation of cinnamic acid are given. The first titrations (zero time) were carried out as soon as possible after mixing the components. The tubes were kept at 37.5° during the whole experiment.

Since the function $(C-B)$ passes through a maximum, it is evident that too much permanganate has been used up in the con-

trol tubes. This was to be expected because the permanganate concentration is always larger in the control, at any given time, than in the reaction tubes and as a consequence more will be used in the oxidation of the solvent in the control than in the reaction tubes. In order to find the maximum value for the oxygen equivalent from the data observed it will be necessary to interpolate to the maximum.

An algebraic equation which satisfies the loci of the three last points on the flat portion of the velocity curve is

$$y = 4.884 + 0.01146t - 0.000471t^2$$

TABLE II.

Determination of Oxygen Equivalent of Carotene.

The volume of oxalic acid used was 15.0 cc. of 0.1 N × 1.007; the weight of carotene used in each reaction tube was 0.003684 gm. or 6.83×10^{-6} equivalents.

Time, *hrs.*	0.0	1.0	3.9	7.0	23.2
Back titer on reaction mixture (*C*), *cc.*	5.67	8.78	9.20	9.38	9.89
Back titer on control (*B*) *cc.*	3.63	3.48	3.55	3.68	4.27
C − *B*, *cc.*	2.04	5.30	5.65	5.70	5.62
Equivalents of $KMnO_4$	0.000204	0.000530	0.000565	0.000570	0.000562
Equivalents of $KMnO_4$	29.74	77.26	82.36	83.09	81.93
Equivalents of carotene					
Oxygen equivalent, found	14.87	38.63	41.18	41.55	40.96

where y is the number of oxygen equivalents, t is the time in hours, and the constants are calculated from the observed data. Differentiating, we have

$$\frac{dy}{dt} = 0.01146 - 0.000942t$$

Setting this differential equal to 0 and solving for the t at which y is a maximum, we have $t_{max.} = 12.15$. Substituting in the original equation and solving for $y_{max.}$ we find $y_{max.} = 4.97$, a value very close to the theoretical 5.00.

The observations on carotene are tabulated in Table II. These

values were obtained by exactly the same analytical methods as those of cinnamic acid. The carotene used was obtained from the extraction of carrots, its melting point was 172° (uncorrected).

Interpolating to the maximum value for the oxygen equivalent in the same manner as with cinnamic acid we find

$$y = 40.147 + 0.272t - 0.01015t^2$$

$$\frac{dy}{dt} = 0.272 - 0.0203t$$

$$t_{max.} = 13.41 \text{ and } y_{max.} = 41.97$$

which is the oxygen equivalent of carotene, a value which is identical with that found by Escher within the experimental error.

SUMMARY.

1. A method has been developed by which the *oxygen equivalent* of an organic substance may be determined in pyridine solution.

2. The oxygen equivalent of cinnamic acid has been found to be 4.97 in agreement with the theoretical value of 5.00.

3. The oxygen equivalent of carotene was found to be 41.97, which confirms that previously reported by Escher, 42.

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THE METABOLISM OF SULFUR.

XVII. THE RATE OF OXIDATION OF INGESTED CYSTINE IN THE ORGANISM OF THE RABBIT.

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After oral administration, cystine is rapidly oxidized and its sulfur excreted by the kidneys, for the most part as inorganic sulfates. With the usual amounts fed, from 75 to 90 per cent of the sulfur present in the cystine molecule is excreted in oxidized form by the kidneys within the 24 hour period immediately following its ingestion. Oxidation of the sulfur after parenteral administration of cystine, particularly after its intravenous injection, does not appear to occur as readily as after feeding.

The experiments of Blum (1), in which cystine was administered orally and intravenously to dogs, are usually cited in this connection. Total sulfur and "labile" sulfur were determined in the urine and the excretion of cystine was considered as having been demonstrated if the sulfur split off as sulfide by boiling with alkali ("labile" sulfur) (2) showed an increase either in absolute amount or in relation to the amount of total sulfur. Large amounts of cystine (4.5 to 9.6 gm.) when fed were almost completely utilized or at least no significant increase in the "labile" sulfur of the urine was observed. Similar results were obtained with dogs poisoned with phosphorus. If however the cystine were introduced directly into the peripheral circulation (jugular or crural vein), cystine was excreted in the urine in such quantities that separation as the characteristic hexagonal plates occurred. When cystine was injected slowly into the mesenteric vein, it could not be identified in the urine, nor was the "labile" sulfur increased significantly. Blum suggested that these differences in the utilization of cystine after introduction into the mesenteric and peripheral circulations

might have been due to a specific influence of the liver on cystine metabolism, possibly to the retention of cystine in the liver for synthesis of taurine. It should be pointed out however that the two series of experiments are not comparable, since in the peripheral intravenous injections, 1.4 and 1.5 gm. of cystine were injected into dogs of 9 and 13 kilos body weight, while in the other series, 1.4 gm. of cystine were introduced into the mesenteric veins of dogs of 30 and 31 kilos body weight. Thus the dose per kilo of body weight in the first series was more than double that of the second series. Moreover in none of the injection experiments was the total sulfur of the urine increased as compared with the normal days. Hence little evidence of the actual utilization or oxidation of the cystine is presented. This failure to observe any increase in the sulfur content of the urine after the injection of cystine is particularly significant in view of the more recent demonstration of the nephrotoxic action of cystine when administered in large amounts (3–7).

The present study was undertaken in an attempt to learn more about the mechanism of the oxidation of ingested cystine, the rate at which oxidation of the sulfur takes place, and the organs concerned in its metabolism. Cystine was given by mouth or subcutaneously to normal rabbits and the urine collected at 3 to 4 hour intervals and analyzed in order to determine the rate as well as the extent of oxidation, and the excretion of unchanged cystine. Similar studies carried out on rabbits whose livers had been injured by the injection of hydrazine will be reported in another paper.

EXPERIMENTAL.

Rabbits, maintained upon a stock diet of 50 gm. of oats and 75 gm. of cabbage daily with water *ad libitum*, received cystine or an ester of cystine either by mouth or subcutaneously. The cystine was usually given at 8 a.m. and the day's food supply was placed in the cage at 5 p.m. This procedure gave ample time for the absorption of cystine from the alimentary tract. The urine was removed by gentle pressure on the abdominal wall. The urine was collected in varying periods as indicated in Tables I to III.

Sulfur partitions were made according to the benzidine volumetric method of Fiske (8), with the use of Jena glass filter

crucibles (No. 10G 3/<7) instead of the paper pulp filters recommended by Fiske. Nitrogen was estimated by the Gunning modification of the Kjeldahl method, creatinine by Folin's micro method, and cystine by the colorimetric method of Looney (9). Okuda (10), who has made a critical study of the Looney method for cystine as applied to urine, concluded that "sodium sulphite itself reacts with the uric acid reagent to give a faintly blue color, and some urinary constituents other than uric acid give a different intensity of color whether in the presence or absence of sodium sulphite. Consequently Looney's method gives higher results especially in urines poor in uric acid." Looney's method according to Okuda gave cystine values from 3 to 10 times as high as those obtained on the same urine samples by Okuda's micro iodine method, the differences not being constant in the various urine samples studied. The cystine values presented in our Tables I to III may, therefore, be higher than the true values, since rabbit urine does not contain any significant amounts of uric acid.

An examination of the data (Tables I to III) would indicate that three factors are significant in determining the rate of elimination of the products of cystine metabolism after its entrance into the system; *i.e.*, degree of absorption, rate of oxidation, and rate of excretion by the kidneys. The amount of absorption in a given period, particularly after subcutaneous injection of cystine, would appear to vary considerably. Although the solutions of cystine injected were always carefully neutralized, in some cases marked edema was produced at the site of injection within a few hours. For this reason oral administration gave most satisfactory results.

In the experiments with Rabbit 14 (Table I), cystine was first given by subcutaneous injection and was evidently very rapidly absorbed from the site of injection, so that the oxidative capacity was exceeded and unchanged cystine was eliminated by the kidneys. 3 hours after the injection, the urine obtained from the bladder contained a heavy precipitate of cystine crystals, and on analysis was shown to contain 137.8 mg. of cystine. An oliguria then developed despite the fact that water was given by stomach tube. As a result, there was not only a failure to excrete the products of oxidation of the ingested cystine, but the normal

TABLE I.

Partition of Sulfur in Urine after Cystine Administration.

Rabbit 14; weight 2.6 kilos.

Day of experiment.	Length of period.	Creatinine.	Total S.	Total SO_4-S.	Inorganic SO_4-S.	Ethereal SO_4-S.	Organic S.	Cystine.	Cystine S.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	3		16.8	12.5	11.2	1.3	4.3		
	3		10.6	7.9	7.9	0.0	2.7		
	18		93.0	78.6	71.0	7.6	14.4		
	24		120.4	99.0	90.1	8.9	21.4		
2	3		9.3	7.1	6.4	0.7	2.2		
	3*		76.8	18.7	18.6	0.1	58.1	137.8	36.8
	6		5.8	1.5			4.3	11.2	3.0
	12†		39.7	31.0	30.7	0.3	8.7	16.1	4.3
	24		131.6	58.3			73.3		
3‡	7		47.6	43.6	42.2	1.4	4.0	8.9	2.4
	17		203.0	181.7	175.7	6.0	21.3	24.6	6.6
	24		250.6	225.3	217.9	7.4	25.3	33.5	9.0
4	6	28.6	13.1	9.7	5.5	4.2	3.4	8.6	2.3
	18	82.4	113.2	102.2	89.9	12.3	11.0	18.0	4.8
	24	111.0	126.3	111.9	95.4	16.5	14.4	26.6	7.1
5	6	27.8	21.4	16.0	14.8	1.2	5.4	7.7	2.0
	18	62.5	107.7	93.6	86.3	7.3	14.1	15.7	4.2
	24	90.3	129.1	109.6	101.1	8.5	19.5	23.4	6.2
6	6	27.8	21.4	16.4	14.8	1.2	5.4	7.7	2.0
	18	62.5	107.7	93.6	86.3	7.3	14.1	15.7	4.2
	24	90.3	129.1	109.6	101.1	8.5	19.5	23.4	6.2
7	6§	40.4	123.4	114.6	104.4	10.2	8.8	12.9	3.4
	6	22.5	156.4	141.4	137.4	4.0	15.0	14.3	3.8
	12	53.0	261.4	242.1	233.8	8.3	19.3	12.4	3.3
	24	115.9	541.2	498.1	475.6	22.5	43.1	39.6	10.5
8	6	19.1	40.0	33.6	32.2	1.4	6.4	5.7	1.5
	18	93.0	135.5	112.1	104.6	7.5	23.4	24.8	6.1
	24	112.1	175.5	145.7	136.8	8.9	29.8	30.5	7.6
9	24		166.4	119.5	112.5	7.0	46.9	25.0	6.7

* Cystine 1.94 gm. (sulfur = 0.517 gm.) given subcutaneously as the sodium salt; 50 cc. of water by mouth.

† Casts in urine. Urine volume very scanty (22 cc.). Kidney injury?

‡ The experiments were performed on consecutive days except that an interval of 50 days elapsed between the 3rd and 4th days designated.

§ Cystine 2.0 gm. (sulfur = 0.534 gm.) given by mouth as the sodium salt.

TABLE II.

Partition of Sulfur in Urine after Cystine Administration.

Rabbit 16; weight 2.75 kilos.

Day of experiment.	Length of period.	Total S.	Total SO_4-S.	Inorganic SO_4-S.	Ethereal SO_4-S.	Organic S.	Cystine.	Cystine S.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	3	10.7	9.5	7.1	2.4	1.2	6.9	1.9
	3	3.8		2.9			6.1	1.6
	18	123.6	111.1	100.9	10.2	12.5	12.5	3.3
	24	138.1		110.9			25.5	6.8
2	3	15.7	13.1	7.2	5.9	2.6	5.9	1.6
	3	9.1	8.3	6.1	2.2	0.8	4.1	1.1
	18	111.0	100.4	79.6	20.8	10.6	7.5	2.1
	24	135.8	121.8	92.9	28.9	14.0	17.5	4.8
3	3*	31.5	17.0	15.6	1.4	14.5	38.4	10.4
	3	71.2	48.9	41.5	7.4	22.3	48.3	12.9
	18	261.0	220.3	201.2	19.1	40.7	20.3	5.4
	24	363.7	286.2	258.3	27.9	77.5	107.4	38.7
4	3	21.7	17.4	15.9	1.5	4.3	6.8	1.8
	3	14.4	11.1	10.0	1.1	3.3	5.7	1.5
	18	108.2	94.4	87.8	6.6	13.8	19.5	5.2
	24	144.3	122.9	113.7	9.2	21.4	32.0	8.5
5	6	16.5	15.5	14.3	1.2	1.0	6.7	1.8
	18	130.4	113.4	107.3	6.1	17.0	23.1	6.2
	24	146.9	128.9	121.6	7.3	18.0	29.8	8.0
6	9	23.4	19.8	18.4	1.4	3.6	10.9	2.9
	15	113.1	99.6	96.0	3.6	13.5	15.5	4.1
	24	136.5	119.4	114.4	5.0	17.1	26.4	7.0
7	3	5.9		3.7			4.8	1.3
	6	18.5	14.2	12.6	1.6	4.3	7.9	2.1
	15	116.9	101.6	89.3	12.3	15.3	13.5	3.6
	24	141.3		105.6			26.2	7.0
8	3†	9.9	7.4	7.2	0.2	2.5	9.0	2.4
	3	26.3	22.8	20.9	1.9	3.5	11.0	2.9
	3	57.2	53.2	52.6	0.6	4.0	5.6	1.5
	15	283.7	252.5	239.4	13.1	31.2	25.4	6.7
	24	377.1	335.9	320.1	15.8	41.2	51.0	14.5

TABLE II—*Concluded.*

Day of experiment.	Length of period.	Total S.	Total SO_4-S.	Inorganic SO_4-S.	Ethereal SO_4-S.	Organic S.	Cystine.	Cystine S.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
9	6	20.4	17.9	17.1	0.8	2.5	5.2	1.4
	18	117.0	113.1	106.1	7.0	3.9	11.7	3.1
	24	137.4	131.0	123.2	7.8	6.4	16.9	4.5
10	24	128.8	107.1	99.4	7.7	14.7	16.5	4.4

* Cystine 0.812 gm. (sulfur = 0.218 gm.) given subcutaneously as the sodium salt; 50 cc. of water by mouth.

† Cystine 1.244 gm. (sulfur = 0.332 gm.) given subcutaneously as the sodium salt; 50 cc. of water by mouth.

excretion of urinary sulfates was also much depressed in subsequent periods. The urine obtained next morning contained protein and casts, evidence of definite kidney injury. During the first 24 hours following the injection, the total sulfur excretion was increased about 13 mg., but almost 60 per cent of the total sulfur was eliminated in the 3 hour period immediately following the administration of the cystine, for the most part as organic sulfur. The amount of oxidized (sulfate) sulfur excreted was also increased about 3-fold in this same 3 hour period. In this case, as in many others, examination of the analytical results of a single 24 hour period fails to reveal the true picture; *i.e.*, overwhelming of the oxidative function, followed by a depression of the normal excretion. In the succeeding 24 hours, excretion of oxidized sulfur was increased above the normal, so that the extra sulfur of the 48 hour period after injection of cystine accounted for about 27 per cent of the cystine sulfur administered. This represents the lowest rate of excretion and the lowest recovery of sulfur after cystine injection which we have observed in these experiments.

In marked contrast with these results are the data of a second experiment carried out nearly 2 months later on the same rabbit, after apparent recovery from the kidney injury, as evidenced by the absence of protein and casts from the urine. Approximately the same amount of cystine was given as earlier but the administration as the sodium salt was oral. It will be noted (Table I) that

TABLE III.

Partition of Sulfur in Urine after Administration of Cystine and Cystine Diethyl Ester Hydrochloride.

Rabbit 17; weight 2.4 kilos.

Day of experiment.	Length of period.	Creatinine.	Total S.	Total SO_4-S.	Inorganic SO_4-S.	Ethereal SO_4-S.	Organic S.	Cystine.	Cystine S.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	8	28.9	22.7	20.1	19.1	1.0	2.6	5.0	1.3
	16	68.2	50.4	40.8	34.1	6.7	9.6	7.3	2.0
	24	97.1	73.1	60.9	53.2	7.7	12.2	12.3	3.3
2	8	32.9	16.1	12.3	9.6	2.7	3.7	6.3	1.7
	16	68.3	105.1	85.2	76.0	9.2	19.9	13.0	3.5
	24	101.2	121.2	97.5	85.6	11.9	23.6	19.3	5.2
3	4*	17.3	18.1	14.7	14.5	0.2	3.4	17.3	4.6
	4	18.0	53.8	45.4	45.2	0.2	8.4	7.6	2.0
	4	19.7	60.1	54.0	53.5	0.5	6.1	5.0	1.3
	12	51.8	134.3	117.2	110.5	6.7	17.1		
	24	106.8	266.3	231.3	223.7	7.6	35.0		
4	8	31.3	42.4	40.4	35.1	5.3	2.0	6.8	1.8
	16	50.2	131.2	111.6	102.0	9.6	19.6		
	24	81.5	173.6	152.0	137.1	14.9	21.6		
5	24	91.5	115.7	106.9	96.3	10.6	8.8	6.3	1.7
6	24	89.6	91.0	60.8	59.4	1.4	30.2	12.4	3.3
7	24	87.0	84.7	71.6	64.7	6.9	13.1	24.4	6.5
8	4†	15.2	29.9	24.7	23.7	1.0	5.2	7.3	1.9
	4	17.1	75.3	57.4	56.7	0.7	17.9	11.6	3.2
	4	14.7	71.8	59.6	58.7	0.9	12.2	6.4	1.7
	12	42.4	146.5	125.9	120.9	5.2	20.6	13.2	3.5
	24	89.4	323.5	267.6	260.0	7.6	55.9	38.4	10.4
9	8	28.8	43.0	40.2	37.7	2.5	2.8	8.9	2.3
	16	64.4	90.0	81.8	71.1	10.7	8.2	14.2	3.8
	24	93.2	133.0	122.0	108.8	13.2	11.0	23.1	6.1
10	8	28.2	26.1	23.8	19.6	4.2	2.3	7.3	2.0
	16	67.4	83.8	76.2	69.6	6.6	7.6	20.2	5.4
	24	95.6	109.9	100.0	89.2	10.8	9.9	27.5	7.4

TABLE III—*Concluded.*

Day of experiment.	Length of period.	Creatinine.	Total S.	Total SO_4-S.	Inorganic SO_4-S.	Ethereal SO_4-S.	Organic S.	Cystine.	Cystine S.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
11	4‡	13.6	18.3	15.0	14.6	0.4	3.3	7.8	2.1
	4	22.5	76.3	67.8	66.0	1.8	8.5	8.5	2.3
	4	15.7	61.5	57.4	56.6	0.8	4.1	7.5	2.0
	12	46.3	112.7	101.8	92.8	9.0	10.9	12.3	3.3
	24	98.1	268.8	242.0	230.0	12.0	26.8	36.1	9.7
12	8	24.7	35.4	33.0	29.7	3.3	2.4	8.9	2.4
	16	61.7	84.7	74.8	68.2	6.6	10.1	14.8	2.9
	24	86.4	120.1	107.8	97.9	9.9	12.5	23.7	5.3
13	24	95.4	108.7	77.6	74.3	3.3	31.1	17.6	4.7
14	24	84.6	130.4	90.7	85.0	5.7	39.7	20.3	5.4

* Cystine 1.0 gm. (sulfur = 0.267 gm.) given by mouth, suspended in 50 cc. of water.

† Cystine diethyl ester hydrochloride 1.689 gm. (sulfur = 0.267 gm.) given by mouth, in solution in 50 cc. of water.

‡ Cystine 1.0 gm. (sulfur = 0.267 gm.) given by mouth as the sodium salt dissolved in 50 cc. of water.

the amount of cystine eliminated as such in the urine (as indicated both by the direct colorimetric determination and by the organic sulfur) was but little increased over the normal amount, while the urinary sulfates increased notably even in the first 6 hours after ingestion. Cystine fed by mouth was absorbed at such a rate that oxidation was normal, the kidneys were not flooded with cystine, and there was no kidney injury as far as could be determined by the microscopic examination for casts and the absence of a positive test for protein. In this experiment the "extra" sulfur eliminated (almost entirely in the form of sulfates) in the first 24 hours following cystine feeding was about 77 per cent of that ingested, with only a small amount of "extra" sulfur (equivalent to about 8 per cent of the cystine fed), an amount within range of normal variations in sulfur excretion, in the succeeding 24 hour period.

Rabbit 16 (Table II) received cystine subcutaneously twice in smaller amounts than in the experiments just discussed. The

products of metabolism were excreted promptly, especially after the smaller amount, which was wholly eliminated in the first 24 hours (approximately 106 per cent of "extra" sulfur), only about 10 per cent appearing as cystine in the urine although there was a notable increase in the elimination of organic sulfur. Despite the larger amount of cystine introduced in the second experiment with this animal, the urinary cystine was only half of that found after the injection of the small dose and the change in the organic sulfur of the urine as well as the cystine sulfur was less marked. This, considered with the less rapid increase in the oxidized (sulfate) sulfur after the injection of the larger amount of cystine, would indicate a less rapid absorption from the site of injection. The absolute amounts of oxidized sulfur excreted in the first 24 hours were approximately the same in both experiments, though the percentages of recovery were different as the amounts injected were not equal.

The variations in the excretion of unchanged cystine noted in Tables I and II were similar to those obtained in all other experiments in which cystine was administered subcutaneously. When cystine was given by mouth, although there were individual variations in the rate of oxidation, in no case was there any considerable increase in the amount of cystine in the urine, the total excretion for 24 hours as measured by the Looney method rarely exceeding 35 mg. It would appear that unless the system is flooded with cystine, very little escapes through the kidney and that when the normal path of ingestion (oral) is followed, either the rate of absorption is slow or the cystine is taken up by the tissues so rapidly that the amount passing through the kidneys at any one time is not sufficiently great to cause excretion. That such excretion may readily occur when the amount of circulating cystine exceeds the threshold value of the kidney has been demonstrated repeatedly in unpublished experiments by one of us (L.) with Eisler and Lough. After the intravenous injection of 3 gm. of cystine (sodium salt) in Ringer-Locke solution into the marginal ear vein of a 2.6 kilo rabbit, 1.01 gm. of cystine had been excreted in an hour. Similarly 2 gm. of cystine were injected into the femoral vein of a 3 kilo rabbit. Within 10 minutes, the concentration of cystine was such that, on cooling, the urine deposited crystals of cystine. At the end of 30 minutes 507 mg.

of cystine had been excreted in the urine. With smaller amounts of cystine excretion also occurred. Thus, 460 mg. of cystine were excreted in the urine 45 minutes after the injection of 1.04 gm. of cystine into the marginal ear vein of a 2.15 kilo rabbit. Other experiments showed similarly the excretion of large amounts of cystine after intravenous injection.

Rabbit 17 (Table III) was fed cystine twice and also a more soluble form of cystine, cystine diethyl ester hydrochloride. The latter appeared to be absorbed somewhat more rapidly than the cystine, as the excretion during the first 4 hours after feeding was definitely higher than in the cystine experiment. The rate of excretion was greatest during the second and third 4 hour periods, being nearly constant during these 8 hours and reaching a value 3 to 4 times the normal value. The "extra" sulfur excreted within the first 24 hour period was equivalent to 60.9 and 61.6 per cent respectively of the ingested cystine in the two experiments in which cystine was fed. When cystine was fed in suspension rather than as the sodium salt, the elimination of "extra" sulfur continued in the second 24 hour period so that a total of 86.8 per cent of the sulfur of the ingested cystine appeared in the urine within 48 hours. When the cystine was fed as the sodium salt, the total recovery in terms of sulfur was less, 67.5 per cent being excreted in 48 hours. When the ester was fed, the excretion as urinary "extra" sulfur in the 24 and 48 hour periods following feeding were 82.0 and 92.8 per cent respectively. Other experiments have also indicated a greater excretion of "extra" sulfur after the feeding of the more soluble ester. These experiments detailed in Tables I to III are typical of the results we have obtained in studying the rate of elimination and the distribution of sulfur following the administration of cystine.

A few studies were made of the rate of absorption of cystine from the intestine. A normal rabbit of about 2 kilos weight was killed after a fast of 3 days duration. The gastrointestinal canal was removed, the contents washed into a volumetric flask, the protein was precipitated by the addition of 10 per cent sodium tungstate and 0.67 N hydrochloric acid,[1] and the contents of the

[1] Hydrochloric acid was substituted for the usual sulfuric acid, in the hope that it might be possible to determine the partition of sulfur in these filtrates. The presence of variable amounts of hydrogen sulfide made this impracticable.

flask diluted to a liter and filtered. The amount of cystine as determined colorimetrically by the Folin-Looney method (after allowance for the color in the blank to which no sulfite was added) was 72.7 mg., equivalent to 19.4 mg. of sulfur. Rabbit 29 (2.5 kilos) was fed 2 gm. of cystine and was killed 6 hours later. 385 mg. of cystine were present in the alimentary tract, or about 20 per cent of the amount fed. 6 hours after the oral administration of 1 gm. of cystine to Rabbit 33 (1.75 kilos), 33.3 per cent of the ingested cystine was present in the contents of the gastrointestinal canal. In each of these cases, the odor of hydrogen sulfide was noticeable on acidification of the intestinal contents, but was not more marked than in the case of the normal animal which received no cystine. The absorption of cystine would not appear to have proceeded rapidly, particularly since these were all fasted animals and the cystine was fed as the soluble sodium salt.

Oxidation of cystine to sulfate must occur rapidly after absorption from the intestine, since under our experimental conditions, excretion of "extra" sulfate sulfur began within 3 hours with a maximum rate per hour at about the 8th hour after feeding. The rate of excretion increased markedly, frequently reaching 25 to 26 mg. per hour for total sulfur, as compared with a normal rate of 4 to 6 mg. per hour. This markedly increased excretion did not appear to cause acute kidney injury, in so far as could be determined by the examination of the urine for casts or protein, so long as the "extra" sulfur was excreted in oxidized form, but kidney injury was usually associated with any marked increase in the excretion of unchanged cystine.

Very little of the "extra" sulfate excreted was conjugated. By far the largest percentage of the increase was in the inorganic sulfate fraction. It will be noted that the cystine sulfur of the urine failed to account for all the organic sulfur excreted. The excess may have been due to thiosulfates[2] although qualitative tests on some of the urine samples failed to reveal their presence. The "extra" organic sulfur may also have originated from other intermediary products of cystine oxidation; *e.g.*, cysteic acid or taurine.

While the greater part of the "extra" sulfur was excreted during

[2] Blum (1) has stated that thiosulfates are not present in the urine of the Herbivora, although of common occurrence in the urine of the dog and cat.

the first 24 hours, the sulfur content of the urine of the second 24 hours following administration of cystine was often somewhat greater than normal. By the 3rd day, the excretion had returned to or was even lower than the normal figure. The increase in sulfur excretion the 2nd day after feeding as noted by Sherwin and his associates (11) was seldom observed by us. In only one instance, after the subcutaneous injection of cystine, was there any definite rise and this was a slight one. Sherwin noted that the urine of the 2nd day after feeding cystine derivatives sometimes contained less sulfur than the control days, which he believed might indicate a definite disturbance of body processes. He also considered that some of the sulfur excreted after the ingestion of cystine and cysteine compounds might be derived from body tissues. Inasmuch as detailed data are not available, it is difficult to interpret these facts, particularly in view of the marked variations in sulfur excretion in animals even on a constant diet.

It is believed that the results presented in this paper, typical of those obtained with a considerable number of animals, afford some explanation of the variations in sulfur elimination frequently observed after the administration of cystine. Sufficient emphasis has not been placed previously on the variations in the rate of absorption, on the possible injury to the kidneys, which might slow up the excretion (as in Table I of the present series) even though absorption and oxidation had occurred, or on the desulfurization of the cystine molecule by the action of the microorganisms of the intestine with the elimination of part of the hydrogen sulfide or mercaptan formed through the feces. A complete picture of the extent of oxidation of ingested cystine and the excretion of the products of oxidation, together with the time relationships involved, is possible only when all these factors, some difficult to evaluate, are considered.

SUMMARY.

1. The absorption of moderate amounts of cystine from the alimentary canal of normal rabbits does not appear to occur with sufficient rapidity to overwhelm the oxidative capacity of the organism. The oxidation of the cystine absorbed and the excretion of the products of oxidation after oral administration proceed rapidly, from 60 to 85 per cent of the sulfur administered as

cystine being eliminated within 24 hours, largely as inorganic sulfates.

2. The course of the metabolism of cystine after subcutaneous injection is variable. If the cystine is rapidly absorbed from the site of injection, the effect is similar to that noted by Blum (1) when cystine was introduced directly into the peripheral circulation; *i.e.*, a considerable part of the cystine is excreted unchanged into the urine. This may be accompanied by kidney injury which may result in a diminished rate of excretion of the products of normal sulfur metabolism. If, however, the cystine is slowly absorbed from the site of injection, the course of the oxidation and excretion of the sulfur is very similar to that observed when cystine is fed by mouth.

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THE BASIC AMINO ACIDS OF WOOL.*

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(Received for publication, December 31, 1929.)

Attention was drawn, in a recent paper from this laboratory (1), to the high proportion of arginine and low proportion of histidine yielded by the keratin of human hair and also to the close analogy between human hair and horsehair with respect to their high yield of arginine. It seemed a matter of some interest to extend our observations to hair from another source to see if this analogy would still hold. We have therefore carried out an analysis of the basic amino acids yielded by wool, employing certain modifications of the silver precipitation method that have been described in previous papers from this laboratory (1, 2).

Marston (3), in a valuable study of the chemical properties of wool, has reported an estimation by Van Slyke's indirect method of analysis of the proportions of the basic amino acids yielded by this tissue. The results were given with some reservation because of the difficulty of obtaining trustworthy data by this method upon a protein that yields a high proportion of cystine. He obtained figures, however, that indicated that the keratin of wool yields 10.2 per cent of arginine, 6.9 per cent of histidine, and 2.8 per cent of lysine. If this analysis is correct wool should form a valuable source of arginine and histidine since worth while yields of both amino acids could be secured from a single protein.

In the present analysis no attempt was made to remove part of the cystine from the hydrolysate by direct crystallization as was done in the analysis of human hair (1). More extensive experience has shown that the cystine present in the histidine frac-

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

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tion can be almost completely removed early in the procedure by precipitation as its copper compound and that no further difficulty from the presence of cystine is then encountered.

The results of the analysis of wool are given in Table I and, with respect to the yield of arginine and lysine, agree fairly well with Marston's data, but the wide difference between the proportions of histidine that we have found and that given by him calls for some comment. The proportions of the three basic amino acids derived from wool agree so closely with those obtained from human hair that we are inclined to believe that a marked analogy exists in the chemical composition of the two

TABLE I.

Basic Amino Acids Yielded by Keratins of Hair.

	Wool (authors).	Wool (Marston (3)).	Human hair (Vickery and Leavenworth (1)).	Horsehair (Vickery and Leavenworth (1)).
	per cent	*per cent*	*per cent*	*per cent*
Histidine	0.66	6.9	0.5	
Arginine	7.8	10.2	8.0	7.6*
Lysine	2.3	2.8	2.5	

* This is a minimal figure and is based upon the hair as weighed without correction for moisture content or ash.

types of keratin.[1] Furthermore other investigators, *e.g.* Argiris (5), who have attempted the determination of histidine in tissues of this type have invariably reported very low proportions of this base. We feel, therefore, that Marston has been led to an erroneous conclusion with respect to the proportion of histidine yielded by wool.

EXPERIMENTAL.

The wool employed in this work had been especially prepared for surgical dressings. It was thoroughly clean and had been sterilized. A sample lost 12.52 per cent in weight when it was extracted successively with ether and absolute alcohol and subsequently dried at 110°, and left 0.81 per cent of ash on ignition.

[1] See in this connection ((4) p. 483).

The nitrogen content calculated ash-, fat-, and moisture-free was 16.57 per cent, the sulfur content was 4.52 per cent, and the fat-free wool contained no phosphorus. Hydrolysis was effected by boiling 198.1 gm., corrected, of wool for 24 hours with 1750 cc. of 8 N sulfuric acid. The hydrolysate was diluted and 95 per cent of the sulfuric acid contained in it was precipitated by barium hydroxide; the barium sulfate was extensively washed by decantation and was then centrifuged.

The solution and washings (70 liters) were concentrated *in vacuo* to 3 liters and the arginine, histidine, and much of the cystine were precipitated together at an alkaline reaction to alizarin yellow (approximately pH 12) as silver compounds. To make sure that as little arginine as possible escaped precipitation, the filtrate from the main precipitate was acidified, concentrated, and a second silver precipitate was taken out at pH 12 at a total volume of 6 liters. The two precipitates were decomposed by hydrogen sulfide and the histidine together with most of the cystine was removed from the solution by precipitation as silver compounds at pH 7.4. The crude histidine-cystine fraction was freed from cystine by boiling with an excess of copper carbonate. After standing several hours the precipitate, which contained the cystine as its copper compound, was filtered off and copper was removed from the filtrate by hydrogen sulfide. In order to insure complete separation of histidine from arginine the solution was concentrated to remove hydrogen sulfide and a second silver precipitation was conducted at pH 7.3. The filtrates from these two histidine precipitations were combined, acidified, concentrated, excess silver was again added, and the arginine silver was precipitated at pH 12. The filtrate, after removal of reagents, was added to the filtrate from the first alkaline silver precipitate to form the main lysine fraction. By these double precipitations washing of the silver precipitates was largely avoided.

The histidine fraction was further purified by precipitation with Hopkins' mercuric sulfate reagent and the solution, obtained after decomposition of the mercury precipitate and removal of reagents, was analyzed by Folin and Marenzi's (6) method for cystine. The small amount found indicated the effectiveness of the treatment with copper carbonate at an earlier stage in the analysis than we have heretofore recommended. A second copper

treatment, while it would undoubtedly have removed most of the remaining cystine, was not carried out because of the possible losses of histidine by adsorption on the precipitates.

The histidine solution contained 0.391 gm. of nitrogen. If 0.026 gm. is deducted for the 0.232 gm. of cystine indicated by the colorimetric method, the solution contained 0.365 gm. of nitrogen presumably belonging to histidine. This is the equivalent of 0.68 per cent of histidine derived from the wool hydrolyzed. Histidine dinitronaphtholsulfonate ("diflavianate") weighing 6.646 gm. corrected, or the equivalent of 0.66 per cent of histidine from the keratin of the wool, was isolated from this solution. The salt contained 8.25 per cent of sulfur, theory 8.17 per cent. It is clear, therefore, that the histidine fraction secured in this analysis was unusually pure and that the chief impurity was cystine.

The arginine fraction contained 6.02 gm. of nitrogen or the equivalent of 9.4 per cent of arginine derived from the wool. Aliquot parts were treated with dinitronaphtholsulfonic acid and the equivalent of 43.36 gm. of the arginine salt containing 6.64 per cent of sulfur (theory 6.56 per cent) were isolated. This is equivalent to 7.8 per cent of arginine derived from the keratin of wool.

The combined lysine fractions were freed from ammonia by distillation in the presence of alcohol at alkaline reaction. Lysine was precipitated as its phosphotungstate in the usual way and converted to the picrate. The first crop, when recrystallized from water, gave needles weighing 9.253 gm. that decomposed at 264–265° in the manner characteristic of pure lysine picrate. The mother liquors were combined, and freed from picric acid; the lysine in them was then precipitated as phosphotungstate and converted to the picrate. Needles weighing 2.304 gm. that decomposed at 256° were obtained. The combined crops of lysine picrate were equivalent to 2.28 per cent of lysine derived from the keratin of wool.

SUMMARY.

The basic amino acids have been isolated from the protein of sheep wool by the modified silver precipitation method. The keratin of the hair of this species yielded 0.66 per cent of histidine,

7.8 per cent of arginine, and 2.3 per cent of lysine and is closely comparable to that of human hair with respect to the yields of these three bases.

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DOES CABBAGE FED TO RABBITS INCREASE SERUM CALCIUM?

A REPLY TO KAPSINOW AND UNDERHILL.

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(Received for publication, November 11, 1929.)

Kapsinow and Underhill (1) in a recent communication on the effect of cabbage on the serum calcium of rabbits refer to a paper published by the author in 1927 (2), in which the observation was recorded that cabbage causes an immediate rise in the serum calcium provided that the initial value is not already high. They failed to confirm this result, and suggest a possible effect due to the withdrawal of the relatively large samples of blood required for serum calcium determinations. In all experiments control animals bled at the same intervals of time and therefore suffering the same degree of hemorrhage showed no rise, but, as a further control, in some of them the hemoglobin was followed at the same time as the serum calcium. The object of the present paper is to present these hitherto unpublished results, and to explain one or two points that were perhaps not clearly emphasized in the original communication.

In the course of a test on the effect of insulin, the hemoglobin of ten rabbits was followed together with the serum calcium at 4 hour intervals for 24 hours. The hemoglobin variation was practically the same for all the rabbits, showing a steady fall to an average of 67 per cent of the initial value. Each time the rabbits were given cabbage there was an increase in the serum calcium and no connection whatever could be observed between the calcium concentration and the effect of hemorrhage.

The above experiment was followed by another in which all conditions except the supply of cabbage were kept constant throughout. Three rabbits were prepared for test by removing cabbage

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from their cages and giving them water instead at 12 noon, no more water being given after 5 o'clock. They were bled at 10 a.m. the following morning and immediately given a liberal supply of cabbage, the remainder being removed after 2 hours. The following values were obtained for the serum calcium and hemoglobin (expressed as percentages of initial values).

Rabbit No.	10 a.m.		12 noon.		2 p.m.		4 p.m.	
	Serum Ca.	Hb	Serum Ca.	Hb	Serum Ca.	Hb	Serum Ca.	Hb
G 4	13.73	100	15.18	104	14.70	96	13.63	91
H 8	13.35	100	14.31	82	12.76	88	12.28	86
I 8	14.21	100	15.57	97	13.73	93	13.24	86

As before, an immediate rise is observed in the serum calcium while the hemoglobin shows a slight fall (except for Rabbit G 4 which shows a very small rise in the first 2 hours).

The result was confirmed on a group of ten rabbits, five of which were given cabbage, while from the other five it was withheld. As this experiment is described in the communication to which Kapsinow and Underhill refer, more details are unnecessary here.

Determinations of serum calcium after the consumption of cabbage were carried out repeatedly on a large number of rabbits and an increase was observed in all experiments except one on a group of ten young rabbits brought in to replace others which had been in use for about 6 months.

That the initial values were higher than those found in any other experiment on the effect of cabbage (the average value was 14.24 mg. per 100 cc. of blood) was put forward as a tentative explanation of this exceptional result. The negative results of the experiment on four rabbits reported by Kapsinow and Underhill cannot however be similarly explained, because their initial values were not very high. There may well be some other factor that would account for the lack of response in their experiment and in that described above, but some confirmation of the suggestion was obtained when the same ten rabbits used 3

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weeks later were given cabbage at the second instead of at the first time interval, and all gave the expected result. This experiment is quoted by Kapsinow and Underhill, but as there is a misprint in one of the averages, and also for the sake of comparison with the above, the figures are recapitulated in Table I.

It will be seen that one of the controls also gave a rise (0.89 mg.) which is greater than the experimental error of the analysis,

TABLE I.

Effect of Cabbage in Checking Fall in Serum Calcium Induced by Hemorrhage.

The results are expressed in mg. per 100 cc. of blood.

	Rabbit No.	9.30 a.m.	11.30 a.m.	1.30 p.m.	3.30 p.m.
Cabbage at 11.30 a.m. to 1.30 p.m.	U 1	13.24	13.04	15.53	14.64
	U 2	14.34	13.14	15.24	14.24
	U 3	14.44	13.84	15.93	14.34
	U 4	14.64	13.94	15.24	13.94
	U 5	13.64	11.05	14.94	13.54
	U 6	14.44	13.84	15.14	14.04
Average		14.12	13.14	15.34	14.12
Change from initial value. Average rise after cabbage, 2.20.			−0.98	+1.22	0
Controls.	U 7	13.04	12.94	13.04	12.05
	U 8	13.54	12.15	13.04	12.35
	U 9	14.24	13.84	12.84	13.04
	U 10	13.14	13.14	11.65	11.55
Average		13.49	13.02	12.64	12.25
Change from initial value			−0.47	−0.85	−1.24

(taken as ±0.5 mg. per 100 cc.) but much less than the average value for those that received cabbage (2.20 mg.).

The "long term" experiments carried out by Kapsinow and Underhill demonstrate clearly that there is no permanent effect of cabbage on the serum calcium, which is in agreement with the observations of the present author, for in all experiments in which an increase was found 2 hours after feeding, a return to the initial or an even lower value was observed by the end of 6 hours.

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SUMMARY.

Further experiments are described to show that the consumption of cabbage produces a definite, though temporary, rise in the serum calcium of rabbits, and that the moderate hemorrhage induced to obtain the necessary blood samples for analysis causes no such rise.

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LONG TIME FEEDING EXPERIMENTS WITH ACTIVATED ERGOSTEROL. I.

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(Received for publication, December 28, 1929.)

In the earliest reference to the action of large doses of irradiated ergosterol, Rosenheim and Webster (1) reported that 10,000 times the minimum antiricketic dose was not lethal to rats. Kreitmair and Moll (2), using "enormous doses," described pathological findings for several species, and observed a species difference in susceptibility. Collazo, Rubino, and Varela (3) observed that rats died within 6 weeks when fed the McCollum high calcium, rickets-producing Diet 3143 plus 50,000 times overdosage of activated ergosterol. The important work of Harris and Moore (4) demonstrated that 1000 times overdosage of vitamin D was harmless to rats, whereas 100,000 times overdosage was rapidly fatal. The agent which produced the characteristic lesions was apparently the activated ergosterol *per se*, and not unchanged ergosterol or decomposition products. Light, Miller, and Frey (5) reported that while 100,000 times overdosage was fatal, 10,000 times had no effect on growth during 6 months. Numerous other papers, mostly quite uncritical, have dealt with the effects of extraordinary doses. In nearly every case the authors failed to determine the potency of the product which they employed. They stated only the quantity of irradiated ergosterol in mg.

It must be accepted that remarkably large doses of activated ergosterol are not immediately toxic, although sufficiently enormous doses do produce serious effects. Up to the present, published experiments have been based on short time tests, covering at the most a few months of life in the rat. It is our purpose to describe the results of tests continued from infancy to old age.

The ergosterol was activated by exposing a solution in arachis

oil to the rays of a mercury vapor quartz lamp. Preliminary tests (thirty-four rats) indicated that there was no great difference in the toxicity of ergosterol irradiated in the oil, in ether under a reflux, or as crystals in air, provided the antiricketic potency of the preparations was the same. These tests also showed that the composition of the basal ration is a factor in the action of ergosterol. In all experiments the ergosterol solution was triturated with the ration, fresh mixtures being made weekly.

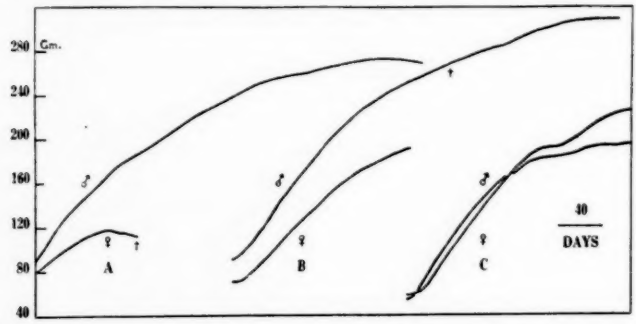

FIG. 1. Composite growth curves of rats on (Curves A) Diet 3143 plus minimum antiricketic dose of activated ergosterol, (Curves B) Diet 3143 plus 100 × overdose of activated ergosterol, and (Curves C) Diet 3143 plus minimum antiricketic dose (0.25 per cent) of cod liver oil.

Experiments with Diet 3143.

In our first long time series the ergosterol solution was standardized to contain 100 times the vitamin D content of average cod liver oil. The basal ration was the rickets-producing Diet 3143 (6). On this diet, without vitamin D, young rats ordinarily die of rickets after several weeks.

Nine rats, 24 days old, were put on Diet 3143. After 19 days, when the rats were rickety, we added to their diet 0.0025 per cent of the ergosterol solution—the very minimum which, in separate tests, would bring about partial healing of rickets. Fig. 1, Curves A, reveals that with this supplement the animals have survived many months. Growth was poor in comparison with that of rats on a normal stock ration (Fig. 2, Curves A). The males are still living after 12 months. One female died after

3 months, and the remainder, being in poor condition, were killed. Autopsy showed rickets.

In a similar series ten rickety rats were given 0.25 per cent of the ergosterol solution; *i.e.*, 100 times the minimum antiricketic dose. After 12 months all are living except one male which developed a respiratory infection and died after 7 months. The growth rate for both sexes (Fig. 1, Curves B) was better than with the minimum dose, but it was decidedly inferior to the growth rate of

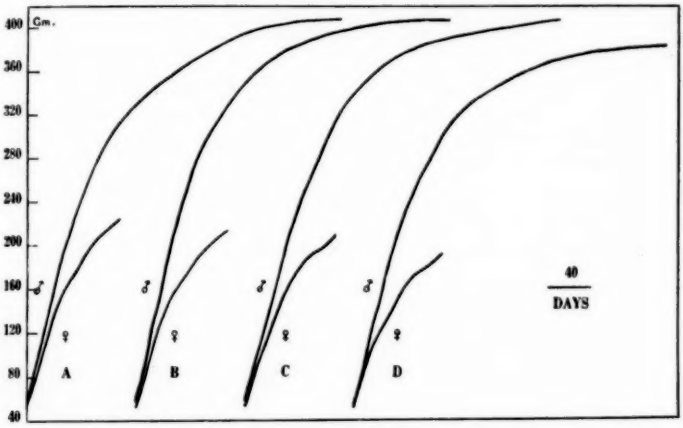

FIG. 2. Composite growth curves of rats on (Curves A) stock diet, (Curves B) stock diet plus minimum antiricketic dose of activated ergosterol, (Curves C) stock diet plus 100 × overdose of activated ergosterol, and (Curves D) stock diet plus non-irradiated ergosterol equal in amount to the activated ergosterol in C.

normal animals. Reproduction was faulty, the young frequently being eaten at birth and in no instance surviving longer than 5 weeks.

In addition to these series, we have made tests (fourteen rats) with concentrated solutions, the animals receiving in Diet 3143 doses from 10,000 to 60,000 times the minimum. The rats showed very wide individual differences in susceptibility to these great doses. During the 1st week some of them lost weight but later developed a partial tolerance. They survived for from 1 to 14 months.

Seven normal rats, 24 days old, weighing from 50 to 60 gm., were put on Diet 3143 supplemented with 0.25 per cent of cod liver oil. Assays had shown this to be the minimum antiricketic level, equivalent to 0.0025 per cent of the standard activated ergosterol solution. Fig. 1, Curves C, indicates that the growth curves of this series were again subnormal and not essentially different from the curves obtained with supplements of activated ergosterol. It follows, therefore, that the depressed growth rate was due to some inherent fault in Diet 3143, and not to the ergosterol or to the oil. The fault was evidently not a lack of vitamin A, since cod liver oil did not correct it.

Experiments with Stock Diet.

As it was manifestly impossible to obtain normal growth and reproduction on Diet 3143, even with added vitamins A and D, we began extensive tests, employing stock diet as the basal ration. For 4 years our breeding colony has been reared on a modified Steenbock stock diet, compounded as follows:

Yellow maize	57
Whole milk powder	25
Linseed oil meal	12
Crude casein	3.7
Alfalfa leaf meal	1.5
"Iodized" table salt	0.4
Calcium carbonate	0.4

To this ration the various additions of ergosterol were made. For each series ten rats were taken from their mothers when 24 days old, and selected to weigh between 50 and 60 gm. Considerable selection was necessary to obtain the requisite uniformity with these superior animals.

Fig. 2 shows the growth curves of rats for 10 months on (Curves A) the normal stock ration, (Curves B) the same plus the minimum antiricketic dose of activated ergosterol, (Curves C) the same plus 100 times overdose of activated ergosterol, and (Curves D) the same plus a quantity of non-irradiated ergosterol equal in amount to the activated ergosterol in Curves C. It is meaningless to state, as is often done in the medical literature, that a certain number of mg. of activated ergosterol were administered. The potency of irradiated ergosterol varies enormously with the condi-

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tions under which activation is performed, values from zero to a million times the potency of average cod liver oil being obtainable. The material used in most of the present work had a potency about 400,000 × prior to dilution. The ergosterol intake of the rats receiving the minimum antiricketic dose may therefore be estimated as $\frac{1}{160,000}$ gm. per day when the food intake was 10 gm.; *i.e.*, the modified diet then contained 1 part of ergosterol to 160,000,000 parts of ration.

It is evident from Fig. 2 that after 10 months the three groups of rats which received ergosterol preparations show growth curves in no way inferior to the normal growth curve with stock ration. Curve D may seem inferior, but one should note that these animals were comparatively small for their age when started. Up to now the general health of the animals has been good, with the following exceptions: On the unsupplemented stock ration one female got a severely infected tail, which was successfully treated with hexylresorcinol. Another female exhibited a transient mammary infection. Of the animals receiving the minimum antiricketic dose of activated ergosterol, and the 100 times overdose, one male in each group has a respiratory infection. One male receiving the untreated ergosterol has shown a lack of equilibrium since the 5th month, and several males have shown minor respiratory infections.

All four groups reproduced normally. The growth curves and general well being of the offspring have been normal. Representative numbers of the second generation have in turn been bred, and their offspring, still receiving the test diets, also appear normal. This phase of our study involved 459 animals. In conformity with our usual practice we reduced all litters to eight.

In another series much larger doses were administered. With the same standard solution of activated ergosterol (100 × the potency of cod liver oil) we ran experiments on two more groups of ten rats each. The first group (Fig. 3, Curves A) received 2.5 per cent of this solution in stock diet (= 1000 × overdosage), and the second group (Fig. 3, Curves B) 10 per cent (= 4000 × overdosage). Ten more rats were used in an arachis oil control (Fig. 3, Curves C), these receiving 10 per cent of oil but no ergosterol.

Fig. 3 shows in all cases a depressed growth rate over 9 months. Between the 1000 × and 4000 × overdoses there is, strangely, little difference, and the depression is but slightly more than in the case of the arachis oil controls. One must conclude, therefore, that the depression is due partly to the activated ergosterol and partly to excessive fat.

In appearance the rats receiving the 1000 × overdose are normal. One male, however, has had a disturbed equilibrium since

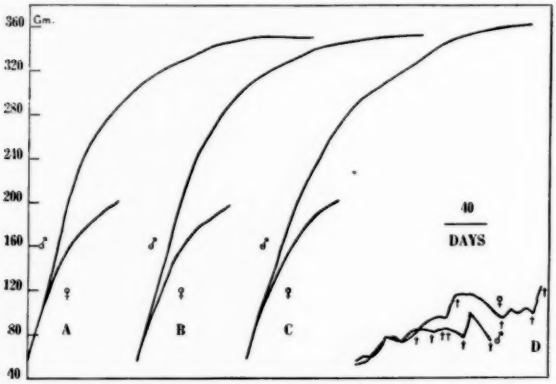

FIG. 3. Composite growth curves of rats on (Curves A) stock diet plus 1000 × overdose of activated ergosterol, (Curves B) stock diet plus 4000 × overdose of activated ergosterol, (Curves C) stock diet plus 10 per cent of arachis oil, and (Curves D) stock diet plus 40,000 × overdose of activated ergosterol.

the 3rd month. One female receiving the 4000 × overdose developed respiratory trouble and diarrhea after 8 months. One male on the arachis oil control developed respiratory trouble after 7 months. All the arachis oil controls and the males on 4000 × overdosage are normal in appearance after 9 months, but the females which received 4000 × overdosage are disheveled and weak, and are losing weight.

The rats on 1000 × overdosage have reproduced normally, and their young have developed at a normal rate. Those on 4000 × overdosage have not done so well. Each of the five females has

been mated three times (with males of the same group). One of these females became pregnant twice, bearing eleven and nine young. Two females failed to become pregnant the first time, but bore young the second, with litters of seven and five. Two females bore twelve and three young the first time, but failed the second. The average number in the litter was eight and the average weight of the young at 24 days of age was 28 gm.—about 40 per cent below normal. All the females failed to reproduce following the third mating.

The control rats on 10 per cent arachis oil had come from the same litters originally as the rats on the 4000 × overdose. Reproduction was better. The average number in the litter was nine, and the average weight of the young at 24 days of age was 42 gm. —only slightly below normal.

This phase of our study involved 231 animals, and it shows that *the effects of overdosage with activated ergosterol become distinct when about 4000 times the minimum antiricketic dose is administered over an extended period.*

Fig. 3, Curves D, illustrates the effect of an overdosage of 40,000 times. The ten rats comprising this test were given the same basal ration, *i.e.* stock diet, as were those in the preceding series. Since it was impossible to administer a quantity of the activated ergosterol solution of standard strength (100 × cod liver oil) sufficient to obtain 40,000 × overdosage, we used a special arachis oil solution that was 1000 times more potent than cod liver oil. 10 per cent of this extremely potent preparation was employed. It is evident from Fig. 3, Curves D, that decidedly toxic effects were produced. After about 2 months the animals began to die, and none of them survived longer than 6 months. They presented the picture of hypervitaminosis described by Collazo, Rubino, and Varela, and others.

Experiments on the Effect of Added Ca and P.

It is particularly interesting to compare the effects of this enormous dose on rats receiving stock diet, with the effects of the same dose given in conjunction with an excess of calcium or phosphorus. Fig. 4, Curves A, illustrates that normal growth obtains with stock ration plus 3 per cent additional calcium carbonate.

Fig. 4, Curves B, shows that with stock ration plus 3 per cent calcium carbonate plus 10 per cent arachis oil the growth rate is depressed slightly, just as it was in the case of stock ration with 10 per cent of this oil. Fig. 4, Curves C, shows that when 10 per cent of the highly potent 1000 × solution (40,000 × overdosage) was added to the stock ration containing the extra 3 per cent of $CaCO_3$, the toxic effect of the activated ergosterol was very marked. The

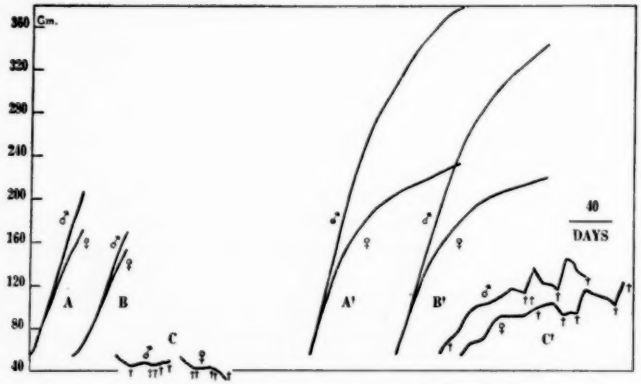

FIG. 4. Composite growth curves of rats on (Curves A) stock diet plus 3 per cent of calcium carbonate, (Curves B) stock diet plus 3 per cent of calcium carbonate plus 10 per cent of arachis oil, (Curves C) stock diet plus 3 per cent of calcium carbonate plus 40,000 × overdose of activated ergosterol, (Curves A′) stock diet plus 3 per cent of disodium phosphate, (Curves B′) stock diet plus 3 per cent of disodium phosphate plus 10 per cent of arachis oil, and (Curves C′) stock diet plus 3 per cent of disodium phosphate plus 40,000 × overdose of activated ergosterol.

rats lost weight from the 1st day, and all died within 7 weeks. They presented Collazo's picture in its most wretched form. In a repetition of this experiment, a female near death was returned to normal stock ration. It is most interesting to note that this rat recovered with great rapidity, and has borne an excellent litter of twelve young.

In Fig. 4, Curves A′, B′, and C′ correspond to Curves A, B, and C, the experiment being conducted exactly as before, except that disodium phosphate, $Na_2HPO_4 \cdot 12H_2O$, was used instead of calcium

carbonate. Again it is evident that the salt addition itself had no effect on growth, and that the 10 per cent addition of arachis oil slightly depressed the growth. When both phosphate and the 40,000 × overdose of activated ergosterol were used, results no different from the earlier experiment without phosphate (Fig. 3, Curves D) were obtained in the case of the females. The males, however, appeared to be slightly protected. Thus we see that while the toxic effect of enormous doses of activated ergosterol is

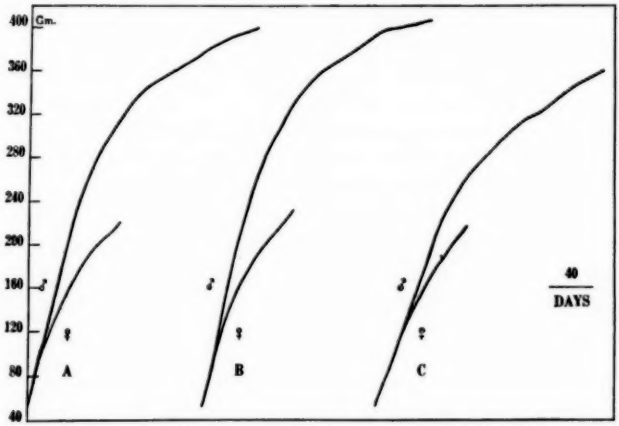

FIG. 5. Composite growth curves of rats on (Curves A) stock diet plus minimum antiricketic dose (0.25 per cent) of cod liver oil, (Curves B) stock diet plus 10 × overdose (2.5 per cent) of cod liver oil, and (Curves C) stock diet plus 40 × overdose (10 per cent) of cod liver oil.

accentuated by additional calcium, an addition of phosphate results in no potentiation, and may possibly lessen the effect of the drug.

Experiments with Stock Diet and Cod Liver Oil.

For purposes of comparison, we have made three sets of tests with stock ration supplemented with cod liver oil. The oil was a medicinal Newfoundland product of average potency, which was carefully assayed, and had served as the standard of potency for preparing the activated ergosterol solutions. Three groups of ten

rats each were given 0.25, 2.5, and 10 per cent of this oil in their diet. The growth curves are shown in Fig. 5. It would seem that the curves for the minimum antiricketic dose of cod liver oil (0.25 per cent), and for 10 × overdosage (2.5 per cent), are similar to those for the unsupplemented stock ration and for the lower levels of activated ergosterol (Fig. 2). However, a 40 × excess (10 per cent) of cod liver oil depressed the growth rate distinctly—to the same degree as did 10 per cent of arachis oil, and slightly less than did 4000 times excess of activated ergosterol (Fig. 3). To explain the growth-depressing action of cod liver oil and arachis oil it is not necessary to postulate a specific toxic principle, inasmuch as the effect can well be associated with the unusual fattiness of the ration.

After 7 months the general appearance of the rats receiving cod liver oil is good. Two males on 0.25 per cent have recently developed respiratory infections. Except for one female on 10 per cent oil, which died in labor, all three groups reproduced normally. The offspring of the rats receiving 0.25 and 2.5 per cent oil have grown at the normal rate. The offspring of the 10 per cent group were inferior, their average weight at 24 days being 37 gm.—about 18 per cent below normal. These observations on reproduction involved 241 young.

Throughout our experiments group consumption records were kept. They revealed no fact of significance except that less food was consumed by the animals receiving strongly toxic preparations and 10 per cent of cod liver oil.

Experiments on Protection Against Rickets.

With so many second and third generation rats at our disposal, we made use of the opportunity to study the influence of activated ergosterol in the mother's diet on resistance to rickets in the young. In the following experiments 200 rats were used. Methods of investigation included the line test (200 rats), ash determinations on alcohol-extracted femurs (56 rats), and blood analyses (80 rats). Parallel determinations showed that there is little choice in the three methods, the simple line test giving results just as significant as the more complicated analytical procedures. Group averages showing rickets by the line test on tibias gave 33 per

cent ash in femurs, 14 mg. per cent Ca in serum, and 2 mg. per cent P in serum.

Omitting a description of the somewhat intricate details of the investigation, we have summarized the results as follows:

Group 1.—Parents on normal stock diet. Young nursed by their own mothers. Young put on rickets-producing diet. Young developed rickets.

Group 2.—Parents on normal stock diet. Young nursed by foster-mother from birth to weaning, the foster-mother receiving minimum antiricketic dose of activated ergosterol in stock diet. Young put on rickets-producing diet. Young developed rickets.

Group 3.—Parents on stock diet plus 100 × overdose of activated ergosterol. Young nursed by their own mother for 3 days, then by foster-mother receiving normal stock diet. Young put on rickets-producing diet. Young developed rickets.

Group 4.—Parents on stock diet plus 1000 × overdose of activated ergosterol. Young nursed by foster-mother from birth to weaning, the foster-mother receiving normal stock diet. Young put on rickets-producing diet. Young developed rickets.

Group 5.—Parents on stock diet plus 1000 × overdose of activated ergosterol. Young nursed by their own mother for 3 days, then by foster-mother receiving normal stock diet. Young put on rickets-producing diet. Young developed only slight rickets.

Group 6.—Parents on normal stock diet. Young nursed by foster-mother during first 4 days, the foster-mother receiving 1000 × overdose of activated ergosterol in stock diet. From 4th day to weaning young returned to their own mother on normal stock diet. Young put on rickets-producing diet. Young developed only slight rickets.

Group 7.—Parents on stock diet plus 100 × overdose of activated ergosterol. Young nursed by their own mother for 16 days, then by foster-mother receiving normal stock diet. Young put on rickets-producing diet. Young developed only slight rickets.

One of us has previously given evidence (7) that the Greenland seal, living on a strongly antiricketic diet of fish, does not transfer vitamin D to the young *in utero*. The present experiment is another example, *a fortiori*, of the same phenomenon. It would appear also that no large amount of activated ergosterol is transmitted through the milk, although nursing rats did obtain sufficient ergosterol for partial protection when their mothers were given great overdoses.

Interesting in this connection is the observation by Hess and Weinstock (8) that vitamin D from cod liver oil was not transferred through the placenta or milk in sufficient amount to protect the

young. Apparently it is a general rule that vitamin D is not transmitted through the placenta, and not through the milk unless very large amounts are administered—by mouth as in our experiments, or by irradiating the mother (9–11).

The investigations which we have reported involved some 1200 rats, including second and third generations. The work covered the period from infancy to late maturity. It is our intention to continue the study on the same original rats, keeping them under observation until they die. We shall later report on the blood chemistry and tissues changes.

SUMMARY.

1. In long time experiments activated ergosterol administered to rats in doses 100 times greater than the minimum antiricketic level showed no effect on general appearance, growth, reproduction, or resistance to respiratory infections. 1000 times overdosage was just perceptibly harmful, 4000 times overdosage definitely injurious, and 40,000 times overdosage strongly toxic.

2. The toxic action was potentiated by calcium carbonate; unaffected or slightly lessened by disodium phosphate.

3. No significant quantity of activated ergosterol was received by rats *in utero*. Nursing rats received in their milk a small amount of vitamin D when the mothers were given enormous overdoses.

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THE BEHAVIOR OF CYSTINE WITH SILVER SALTS.*

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If cystine is dissolved in a minimal amount of dilute sulfuric acid and treated with an excess of silver sulfate solution a turbidity immediately forms. When dilute alkali is added a flocculent precipitate separates and, if this is removed after neutralization of the solution to pH 5 to 6, only an insignificant proportion of the original cystine can be demonstrated in the filtrate by colorimetric tests. Analyses of the amorphous precipitate indicate that the composition is somewhat variable but the chief component appears to be a compound of the formula $C_6H_{10}N_2S_2O_4Ag_2 \cdot Ag_2SO_4$. The significance in protein analysis of this behavior of cystine has been discussed in a recent paper (1) from this laboratory.

Preparation of the Amorphous Silver Precipitate.

A solution of 2.000 gm. of cystine in 100 cc. of water was prepared by the addition of the minimal amount of dilute sulfuric acid; an excess (approximately 600 cc.) of boiling saturated silver sulfate solution was added and the mixture was chilled to 10°. Sodium hydroxide (0.8 N) was then added drop by drop with rapid mechanical stirring until the reaction of a small sample of the clear fluid reached pH 6.0. The temperature was maintained below 10° throughout as dark products were invariably obtained at higher temperatures. The pale yellow precipitate was centrifuged off and washed at least four times, or until free from sulfate, with

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C. We acknowledge with pleasure the benefit derived from the discussion of this problem with Professor R. D. Coghill of Yale University.

ice water, then twice with absolute alcohol, and finally with ether. It was thoroughly dried in a vacuum desiccator over calcium chloride and sulfuric acid; yield approximately 5 gm.

A precipitate that probably contains a compound analogous to this silver sulfate compound can be prepared by neutralization of a dilute nitric acid solution of cystine that contains an excess of silver nitrate. Preparations secured in this way have not yet been subjected to detailed study.

The amorphous silver precipitate, when decomposed by hydrochloric acid, yielded a solution that reacted strongly with sodium nitroprusside in the manner typical of *cysteine*. Sullivan's (2) highly specific naphthoquinone test for cysteine was likewise found to be positive. The precipitate therefore contained a compound of cysteine and silver and it became necessary to determine the relative proportions of cysteine and cystine in it. An attempt to employ colorimetric methods for this purpose did not give satisfactory results owing to the difficulty of preparing standards.

A study of the optical rotation of known cystine-cysteine mixtures indicated that simple interpolation between the specific rotations of cystine and cysteine may be used to calculate the relative proportions of the two constituents of the mixture with sufficient accuracy for the purpose in hand. The specific rotation of our cystine, observed in 1 per cent solution in N hydrochloric acid, was $-215°$ (3). Cysteine is dextrorotatory under the same conditions and its specific rotation has been given by Andrews (4) as $+9.7°$; our own observations indicate, however, that the specific rotation is somewhat higher than this. In Table I are given the results of a number of observations, averaging $+15.9°$. Owing to the error of reading the instrument this figure has an uncertainty of $\pm 1.7°$. The total change in optical rotation that occurs when cystine is reduced to cysteine, therefore, amounts, under the specified conditions of observation, to approximately 231°.

Decomposition of the Amorphous Silver Precipitate.

A specimen of the dry amorphous silver precipitate which weighed 1.600 gm. and contained nitrogen equivalent to 0.500 gm. of cystine, was treated with warm 2 N hydrochloric acid in amount sufficient to precipitate the silver and leave an excess of 25 cc. of 2 N acid. The silver chloride was allowed to digest for a short

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time and the solution was then cooled and diluted to 50 cc.; a sample was withdrawn and centrifuged clear. This solution was found to be weakly dextrorotatory; the specific rotation of the nitrogenous substance therein, calculated as cysteine, was +0.48° (0.99 per cent solution from nitrogen determination; 1.07 N acid).

TABLE I.

Specific Rotation of Cysteine in 1 Per Cent Solution in Normal Hydrochloric Acid.*

Cystine.	Tin.	Time of reduction.	Specific rotation (dextro).
gm.	*gm.*	*hrs.*	*degrees*
5	15	66	15.4
1	2	42	17.5
1	4	18	14.7
1	4	42	17.3
5	15	117	14.2
1	4	22	16.6
1	4	45	17.8
1	4	46	15.2
1	4	48	14.8
1	4	48	13.9
1	4	49	17.9
1	4	50	14.5
5	20	48	16.2
Average.....			15.9

* In most of these experiments 1.000 gm. of cystine was dissolved in 25 cc. of 4 N HCl, fine granulated tin was added and allowed to react for the specified time. The solution was diluted with 25 cc. of cold recently boiled water, treated with H_2S, diluted to 100 cc. with cold boiled water, and immediately filtered through a dry paper into a 2 dm. polarimeter tube. The first few drops of filtrate were discarded. Nitrogen was determined in 25 cc. of clear filtrate and the cysteine concentration was calculated. Where 5 gm. of cystine were taken 5 times the specified amounts of reagents were used. Readings of the optical rotation were made on a saccharimeter and were converted to circular degrees by the factor 0.3468.

The solution therefore contained approximately 93 per cent of its nitrogen in the form of cysteine and only 7 per cent as cystine, provided that no significant amounts of other optically active substances were present. In experiments on other preparations calculated specific rotations of −1.5°, 0.0°, and +4.7° were ob-

served under similar conditions. When the amorphous silver precipitate was decomposed by hydrogen sulfide more highly dextrorotatory solutions were secured. Thus in two experiments the specific rotation of the cysteine observed in 1 per cent solution in N hydrochloric acid was +9.8° and +10.1°. Under these conditions therefore about 97 per cent of the nitrogen in the solution apparently belonged to cysteine.

A specimen of the solution that showed a specific rotation of +4.7°, secured by hydrochloric acid decomposition of the precipitate, was subjected to further reduction with tin and 4 N hydrochloric acid; the specific rotation under standard conditions was thereby increased to +12.1°, indicating that the chief impurity in the cysteine solution was cystine.

Recovery of Cystine from the Amorphous Silver Precipitate.

A preparation of the silver precipitate was secured from 5.000 gm. of cystine as described above except that neutralization was effected with barium hydroxide. After careful washing the precipitate was suspended in water and decomposed during mechanical shaking by hydrogen sulfide. The inorganic precipitate was centrifuged off and thoroughly washed with hot water. The solution and washings were concentrated *in vacuo* to 200 cc. without admitting air to the distillation flask. Nitrogen was determined in 10 cc. (0.446 gm. N = 3.853 gm. of cysteine in 200 cc.) and a mixture of 12.5 cc. with 12.5 cc. of 2 N hydrochloric acid was found to have rotation of +0.19$_4$° in a 2 dm. tube. The cysteine concentration of this solution was 0.963 per cent and the acidity, determined by titration of 1 cc. was 1.08 N. The specific rotation of the cysteine was therefore +10.1°. The remainder (177.5 cc.) of the cysteine solution was neutralized with barium hydroxide to pH 7.0 when the solution became violet in color after agitation. The barium sulfate that separated was removed and carefully washed. The clear solution (800 cc.) was aerated until the violet color permanently disappeared. A test of the clear fluid with Folin and Marenzi's reagent (5) and sodium carbonate then gave no blue color, showing the absence of cysteine. The white, well crystallized cystine that had separated was filtered off and dried. It weighed 2.438 gm., contained 11.64 per cent of nitrogen (theory 11.66 per cent), and had a specific rotation of −208°. The filtrate

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was treated with sulfuric acid to the exact removal of barium and the small precipitate of barium sulfate was washed cystine-free with hot water. The filtrate and washings were concentrated to 200 cc. and allowed to stand several days. 0.427 gm. of cystine of 96.8 per cent purity and specific rotation of $-206°$ separated. This crop contained a little barium sulfate. By further concentration and treatment with alcohol two crops weighing 0.088 and 0.051 gm., the last of which was 85 per cent pure (colorimetric), were secured. The mother liquor contained 0.0603 gm. of nitrogen and 0.115 gm. (colorimetric) of cystine. The total recovery of cystine was 3.489 gm. when corrections for the impurity of the

TABLE II.

*Distribution of Nitrogen, Calculated as Cystine, between the Amorphous Silver Precipitate and the Solution from Which It Was Prepared.**

Experiment No.	Silver precipitate decomposed by:	N in cysteine fraction.	Specific rotation of the cysteine.	N in filtrate.	N in organic precipitate.	Loss.
		per cent	*degrees*	*per cent*	*per cent*	*per cent*
6	HCl	78.2	0.0	14.0		7.8
7	HCl	80.3	+1.8	5.8		13.9
8	HCl	71.6	+4.7	9.5		18.9
10	HCl	79.2	+0.88	6.8		14.0
11	H_2S	84.2	+8.8	5.02		10.8
12	H_2S	76.5	+10.1	4.08	16.4	3.0
14	HCl	81.4	−2.4	7.41	6.9	4.3

* In these experiments 5.000 gm. of cystine were taken and neutralization was effected by barium hydroxide.

cystine and the volume of the solution were applied. Of the original 3.853 gm. of cysteine in this fraction, equivalent to 3.826 gm. of cystine, 91.2 per cent was therefore recovered as cystine.

The amorphous precipitate which separates when a sulfuric acid solution of cystine that contains an excess of silver sulfate is neutralized is thus *essentially a compound of silver and cysteine.* Extensive reduction of cystine evidently takes place during the precipitation and a corresponding oxidation of some component of the system must therefore occur. An examination of the distribution of the nitrogen in a number of experiments (see Table II) indicated that between 70 and 85 per cent of the nitrogen of the cystine

taken appeared in the solution obtained by decomposing the silver precipitate, from 4 to 14 per cent usually appeared in the filtrate, while the balance was lost by adsorption on the inorganic precipitates.

Filtrate from the Amorphous Silver Precipitate.

The filtrate from the amorphous silver precipitate was found to contain a mixture of substances. Usually about 1 per cent of the original cystine could be demonstrated to be present by colorimetric methods. Ammonia equivalent to from 1 to 2 per cent of the original cystine nitrogen was invariably present indicating

TABLE III.

Analyses of Preparations of the Amorphous Silver Precipitate Obtained from Cystine.

The theoretical composition was calculated from the formula $(C_3H_5NSO_2Ag)_2 \cdot Ag_2SO_4$.

	Nitrogen.	Sulfur.	Silver.	Sulfate sulfur.	Cystine.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Theory	3.66	12.56	56.33	4.18	31.36
Preparation 1	3.62	11.48	56.41	3.22	
"		11.45	56.65		
" 2	3.45		57.43	3.25	
" 3 a	3.64	11.68	55.68		
" 5	3.84	11.31	57.66		30.9
	3.71				
" 9	3.75	11.22	56.91	3.22	
			56.56		
Average	3.66	11.43	56.76	3.23	30.9

that the oxidation reactions give rise to extensive decomposition of a part of the cystine. Except for the ammonia the whole of the nitrogen was in the amino form but the only definite substance that we have been able to isolate was a small proportion of cysteic acid, amounting in different experiments to the equivalent of from 0.3 to 1.7 per cent of the cystine taken. The presence of this substance indicates that one of the reactions that takes place during the precipitation of the silver compound may be expressed,

$$3\ R{-}S{-}S{-}R + 3\ H_2O \rightarrow R{-}SO_2{-}H + 5\ R{-}SH$$

The equation implies an oxidation of one-sixth and a reduction of five-sixths of the cystine that undergoes this particular reaction and provides an interesting case of simultaneous oxidation and reduction that bears certain analogies to the well known Canizzaro reaction.

Composition of the Amorphous Silver Precipitate.

Analyses of a number of different preparations of the amorphous silver precipitate are given in Table III.

The total sulfur content was determined after oxidation in a Parr bomb. Silver was determined in solutions secured by suspending the sample in water and boiling, with occasional additions of nitric acid, until the turbid solution became clear. At this stage, it is interesting to note, nearly all the sulfur was present as sulfate ion; solution was therefore effected under these conditions at the expense of considerable decomposition of the organic substances present. Sulfate sulfur was determined in solutions secured by warming the sample with dilute hydrochloric acid until decomposition was complete; the silver chloride was then filtered off and sulfate was determined in the usual way in the filtrate. The weight of silver chloride secured in this procedure gave silver determinations that agreed closely with those obtained by the decomposition with nitric acid. Cystine was determined colorimetrically. A sample of 0.3171 gm. of the preparation was treated with 10 cc. of 20 per cent hydrochloric acid and warmed. The precipitate of silver chloride was removed and thoroughly washed, the solution was neutralized to pH 7.0 with sodium hydroxide, was aerated until the nitroprusside test was negative, and was then acidified with 1 cc. of concentrated hydrochloric acid and made to 200 cc. As a control 0.100 gm. of cystine was carried through the same operations, exactly similar amounts of reagents being used and similar amounts for tests being removed. 6.0 cc. of the control solution and of the sample were compared, after developing the color according to Folin's technique with Folin and Trimble's reagent (6), with results that indicated the presence of 0.098 gm., or 30.9 per cent, of cystine in the sample.

It will be noted that the sulfur determinations are in all cases too low and the silver in all save one case too high. A considerable part of the deficit in total sulfur is due to the low proportion of

sulfate sulfur. It is possible to account in part for these discrepancies by assuming the existence in these precipitates of variable proportions of a compound such as $(C_3H_5NSO_2Ag)_2 \cdot Ag_2O$ which should contain 4.09 per cent of nitrogen, 9.36 per cent of sulfur, and 63.02 per cent of silver. That some such substance may be present is suggested by the results secured from Preparation 3. The material designated Preparation 3a was removed at pH 4.0. Subsequent neutralization of the solution to pH 6.0 gave Preparation 3b which contained 10.2 per cent of sulfur and 59.25 per cent of silver. It is perhaps reasonable to suppose that at the less acid reaction the conditions favored the formation of a double compound with silver oxide rather than with silver sulfate.

The amorphous silver precipitate is insoluble in water and in alcohol. The material is soluble in dilute ammonia and yields a yellow solution the optical rotation of which could not be observed in a 2 dm. tube. In a 1 dm. tube with a powerful light source a 1 per cent solution in N ammonium hydroxide was found to be weakly dextrorotatory with a specific rotation of approximately $+25°$. After standing a few hours this solution darkened and became nearly opaque.

The silver precipitate is soluble in dilute sodium sulfite solutions.

The most convenient solvent is dilute sulfuric acid. 0.500 gm. of Preparation 9 was dispersed by rubbing with 10 cc. of 50 per cent (by volume) sulfuric acid. The resulting opaque colloidal solution was then slowly diluted to 50 cc. with water. After standing overnight a little dark precipitate settled out leaving a water-clear solution. The silver content of the clear solution was 0.0565 gm. in 10 cc. It being assumed that the cysteine silver compound present contained 56.2 per cent of silver, the solution had a concentration with respect to this compound of 0.97 per cent. The specific rotation of the silver compound was $+23.9°$ on this basis. The cysteine content of the solution, calculated likewise from the silver upon the assumption mentioned above, was 0.303 per cent. This gives a calculated specific rotation for the cysteine under these conditions of $+77°$, a figure so far removed from the rotation of cysteine in N hydrochloric acid as to suggest that cysteine is present in these solutions in some unusual type of combination.

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Isolation of Cysteic Acid.

A considerable part of the oxidation products of cystine that are produced during the formation of the silver compound are found in the filtrate from the neutralization precipitate. A filtrate, obtained in an experiment in which 5 gm. of cystine had been taken and the neutralization effected with barium hydroxide, was treated with hydrochloric acid, drop by drop, until a filtered test sample showed the presence of a trace of chloride. The sulfuric acid in the solution was then exactly removed by means of barium hydroxide and the faintly acid solution was centrifuged clear and concentrated *in vacuo* to 250 cc. The work of Friedmann (7) has shown that the basic copper salt of cysteic acid is very insoluble in water. The solution, after removal of an aliquot part for nitrogen determination, was therefore boiled for ½ hour with an excess of copper hydroxide and filtered. The clear blue filtrate was concentrated to 5 cc. and treated with about 2 volumes of alcohol. After standing overnight the small blue powdery precipitate was filtered off, dissolved in boiling water, and filtered from a little dark impurity. The solution was evaporated to 15 cc. and treated with 3 volumes of alcohol. The copper salt was filtered off after standing overnight and was washed with water, alcohol, and ether. After being dried at 105° it weighed 0.152 gm., equivalent to 0.0815 gm. of cystine, when corrected for the aliquot previously removed. This is 1.63 per cent of the cystine taken.

The identity of the material as the basic copper salt of cysteic acid described by Friedmann was established by analysis after two more recrystallizations from aqueous alcohol.

5.935 mg.[1] : 3.175 mg. CO_2 and 1.525 gm. H_2O.
7.515 " : 0.382 cc. N at 26° and 754.7 mm.
3.940 " left 1.260 mg. ash after ignition in oxygen.
Found. C 14.58, H 2.87, N 5.76, ash 31.97.
Calculated for $C_3H_7NSO_6Cu$. C 14.47, H 2.81, N 5.63, ash 32.01.

With regard to other components of the mixture of oxidation products little can be said. Ammonia was determined by Nessler's method, the amount found varying in different experiments from the equivalent of 1.3 to 1.8 per cent of the cystine taken. When

[1] The micro analyses were carried out by the Research Service Laboratories, New York.

evaporated *in vacuo*, after removal of cysteic acid and of reagents, these solutions left sirups from which nothing definite has yet been isolated.

Cystine Silver Compound of Neuberg and Mayer.

Neuberg and Mayer (8) in 1905 described a silver compound of cystine that was prepared by dissolving the amino acid in an equivalent of sodium hydroxide and adding an excess of silver nitrate solution. The white precipitate was washed with water, alcohol, and ether, was dried and found to contain 47.93 per cent of silver (theory for $C_6H_{10}N_2S_2O_4Ag_2$, 47.58 per cent) and 6.30 per cent of nitrogen (theory 6.17 per cent). No further description of the substance was given save that it remained colorless when protected from light. We have repeatedly attempted to prepare this compound by employing a small excess over the calculated amount of silver nitrate added all at once, but have invariably secured products which, although white when first precipitated from the ice-cold solution, became more and more yellow during washing with ice water. When attempts were made to prepare it at room temperature the precipitate immediately became yellow. The preparation which corresponded most closely in composition to that of Neuberg and Mayer contained 5.35 per cent of nitrogen and 50.27 per cent of silver. Other preparations contained higher proportions of silver.

The curious progressive change in the color of the moist material which occurred during the washing operations suggested that the cystine silver salt was not stable. In order to discover if the change was due to a reduction of a part of the cystine, 1.089 gm. of the preparation mentioned above, an amount which should contain 0.500 gm. of cystine calculated from its nitrogen content, were treated with sufficient 2 N hydrochloric acid to precipitate the silver and leave an excess of 25 cc. After being warmed for a short time the solution was diluted to 50 cc. and a sample was centrifuged clear. The specific rotation of the nitrogenous substance, calculated as cystine, was $-135°$. The color tests for cysteine were strongly positive. A sample of the solution was neutralized and aerated. Cystine crystallized out. This was redissolved by the addition of hydrochloric acid to the solution which was then concentrated to 1 per cent cystine content and the

acid concentration was raised to normal. The specific rotation of the nitrogenous substance calculated as cystine was then −180°, demonstrating a reoxidation of cysteine to cystine. Other preparations of the silver compound showed similar behavior.

In order to test the effect of time on the apparent transformation of the Neuberg and Mayer silver compound, the precipitation with silver nitrate in ice-cold solution was carried out as before but the suspension was stirred for 1 minute and hydrochloric acid was then added in amount sufficient to precipitate the silver and leave an excess of 25 cc. of 2 N acid. The solution was diluted to 50 cc. and centrifuged. The specific rotation of the nitrogenous substance calculated as cystine was −201°. A similar experiment in which the precipitate was stirred 10 minutes before decomposition yielded a solution containing cystine of calculated specific rotation −192°. Color tests on these two solutions showed that cysteine was present in both, the reactions being much stronger on the second. Since the cystine employed had a specific rotation of −215° these experiments indicate that reduction of the cystine to the extent of approximately 6 per cent and 10 per cent occurred in 1 and 10 minutes respectively under these conditions. The transformation appears to continue even in a dry preparation preserved in a desiccator in the dark. An examination of the above mentioned dry preparation of the Neuberg and Mayer compound 2 weeks after the observation of −135° had been recorded gave a specific rotation of −121°, while 11 weeks still later it had decreased to −90°.

These results suggest that Neuberg and Mayer's silver compound of cystine, in which the silver presumably replaces the hydrogen of the carboxyl groups, has, in our hands at least, only a transitory existence. At our request Dr. A. J. Wakeman of this laboratory attempted to prepare this compound. His first preparation, made by adding 2 mols of silver nitrate somewhat slqwly to the cold alkaline solution of cystine gave a precipitate that, after being washed, was only faintly yellowish. It contained 47.73 per cent of silver (theory 47.53 per cent). His subsequent preparations were, like our own, decidedly colored and contained high proportions of silver (55.0, 52.6, and 55.1 per cent).

Constitution of the Silver Compound Contained in the Amorphous Silver Precipitate Secured from Acid Solution.

A solution of a preparation of the amorphous silver precipitate in dilute sulfuric acid gave a negative nitroprusside reaction and no color developed when it was treated with Folin and Marenzi's (5) phosphotungstic acid reagent after the addition of sodium carbonate. When a little hydrochloric acid was added to the sulfuric acid solution, silver chloride precipitated and both tests were strongly positive on the filtrate.

A solution of the substance in ammonia gave a negative nitroprusside reaction, and a faint positive reaction with the phosphotungstic acid reagent after the addition of sodium carbonate. After the ammoniacal solution was made acid with sulfuric acid both nitroprusside and phosphotungstic acid reagent tests were negative. If, however, the solution was made acid with hydrochloric acid, the filtrate from the silver chloride gave strong positive reactions with both reagents. Still another striking reaction of this material should be mentioned. 1 cc. of a 1 per cent solution in 10 per cent sulfuric acid was treated with sodium sulfite, sodium carbonate, and phosphotungstic acid reagent according to Folin and Marenzi's technique. A faint blue color developed and faded out within 10 minutes. A similar quantity was treated with 4 drops of dilute hydrochloric acid and then, without removing the silver chloride, with the reagents. Again only a trace of color formed and the solution was practically colorless after 10 minutes. A third 1 cc. sample was treated with 4 drops of dilute hydrochloric acid and the silver chloride was removed. When the reagents were then added an intense blue color developed that was roughly equivalent to that produced by 6 mg. of cystine. It was noted that the precipitate of silver chloride in the second test dissolved when the sulfite and carbonate were added previous to the addition of the phosphotungstic acid reagent. It is evident that the silver chloride, when redissolved, reacted with the cystine to form a compound that could no longer reduce the phosphotungstic acid reagent.

The investigation of the specific rotation of the nitrogenous substance btained by the decomposition of the amorphous silver precipitate secured from acid solutions of cystine by neutralization, indicates that this silver precipitate is essentially a compound of

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cysteine. The color reactions show, however, that the characteristic behavior of the R—SH group is no longer shown by the silver compound. It seems probable, therefore, that in this compound the *silver replaces the hydrogen of the R—SH group* rather than that of the carboxyl group. The characteristic compound in the silver precipitate therefore apparently consists of 2 molecules of the silver mercaptide derivative of cysteine combined with 1 molecule of silver sulfate.

The properties of the Neuberg and Mayer silver compound of cystine, on the other other hand, suggest that it is a silver salt of the normal type. Under the conditions that have been employed in our study of this substance, however, a migration of the silver from the carboxyl to the sulfhydroxyl group seems slowly to take place with a corresponding reduction of a part of the cystine to cysteine. We have not as yet investigated the details of this transformation and Neuberg and Mayer's success in preparing a white product of correct silver and nitrogen content indicates that the change may not invariably occur. This view is proposed as a possible explanation of our observations.

Preparation of Cysteine Hydrochloride and Sulfate.

The behavior of cystine solutions in the presence of silver sulfate suggests a convenient and rapid method to prepare cysteine. 5.000 gm. of cystine were dissolved in a minimal amount of dilute sulfuric acid, an excess of boiling saturated silver sulfate solution was added, and the solution was chilled and neutralized slowly with barium hydroxide during mechanical stirring. The precipitate was removed and washed with cold boiled water, was extracted with dilute hydrochloric acid, and finally warmed for a short time with this reagent. The extracts were, if necessary, filtered clear and were concentrated *in vacuo* at 65–70° to a thin sirup. After standing a short time crystals of cysteine hydrochloride separated identical in appearance and behavior with cysteine hydrochloride secured by the reduction of cystine with tin and hydrochloric acid. When removed from the solution and dried these crystals soon became covered with white powdery crystals of cystine.

For some purposes, where the possible presence of a little impurity is not objectionable, the new method of preparation may

present advantages over reduction with tin and hydrochloric acid. A solution containing a high proportion of its nitrogen as cysteine can be prepared in a relatively short time and the presence of hydrogen sulfide with the attendant difficulties, such as the liberation of sulfur, is entirely avoided. Solutions secured in this way, however, contain the sulfuric acid derived from the decomposition of the silver sulfate in the double compound. The presence of this acid accounts for the slow and incomplete crystallization of the cysteine hydrochloride; it may be removed, however, before concentrating the acid solution, without difficulty.

It may also be pointed out that, if the silver precipitate is decomposed by hydrogen sulfide, a solution is obtained that, after concentrating slightly *in vacuo* to remove hydrogen sulfide, contains practically pure cysteine sulfate. A solution so prepared may possess special advantages in some investigations.

Inorganic Precipitates.

We have secured little information regarding the somewhat large proportion of the original cystine nitrogen which is carried down upon the inorganic precipitates.

The mixture of silver sulfide and barium sulfate, obtained from the decomposition of the silver precipitate in one experiment in which 5.000 gm. of cystine had been used, was boiled with water containing a little hydrochloric acid whereby nitrogen equivalent to 0.245 gm. of cystine was extracted and 0.236 gm. of cystine was found to be present in this extract by the colorimetric method. This figure is, however, without doubt a little high since the solution responded feebly to direct tests for cysteine. The precipitate was then dried and ground. Analysis of a sample indicated the presence of nitrogen equivalent to 0.574 gm. of cystine in the whole. The precipitate was then boiled for several hours with 20 per cent hydrochloric acid and an extract was secured that contained nitrogen equivalent to 0.233 gm. of cystine. Colorimetric tests, however, indicated the presence of only 0.067 gm. of this substance. A large part of the nitrogenous substances lost by adsorption on the inorganic precipitates therefore appears to consist of oxidation products of cystine.

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SUMMARY.

Cystine, when dissolved in dilute sulfuric acid and treated with an excess of silver sulfate yields, on neutralization of the solution to pH 6, a precipitate that contains from 70 to 85 per cent of the nitrogen of the cystine taken and consists, in large part, of the *cysteine* compound $(C_3H_5NSO_2Ag)_2 \cdot Ag_2SO_4$. The color reactions of this compound indicate that it is a silver mercaptide derivative of cysteine. The extensive reduction that occurs during its formation is accompanied by an oxidation that results in the production of a mixture of substances among which ammonia and cysteic acid have been identified. Insignificant amounts of cystine are found in the filtrate from the precipitate. The existence of this silver compound accounts for the presence of cystine and cysteine in the histidine fractions secured during the basic amino acid analysis of proteins by silver precipitation methods.

Neuberg and Mayer have described a silver compound of cystine that was prepared from an alkaline solution of the amino acid by precipitation with silver nitrate. It is presumably a normal silver salt. In our hands this compound has been found spontaneously to undergo a partial change to a mercaptide derivative of cysteine.

A new method to prepare solutions that contain a high proportion of their nitrogen in the form of cysteine is described.

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DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

XIII. THE INFLUENCE OF IRRADIATED YEAST ON THE CALCIUM AND PHOSPHORUS METABOLISM OF MILKING COWS.*

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Recent work at this station (1–3) has shown that any of the following sources of vitamin D, such as ultra-violet light applied directly to the animal, cod liver oil ingested with the ration, or alfalfa hay cured with an optimum exposure to sunlight and used as the main roughage, failed to effect a demonstrable influence on the calcium and phosphorus assimilation in cows producing 40 to 50 pounds of milk per day. In the light of unpublished data showing that irradiated yeast is a most abundant source of vitamin D, we were led to study its effect on the calcium and phosphorus metabolism of heavily lactating cows.

It has seemed to us that on a ration of grains, alfalfa hay, and silage, the phosphorus and calcium intake would probably be sufficient if there were at the same time a plentiful supply of vitamin D in the ration, and if this vitamin D were available to the animal. Curiously the ingestion of cod liver oil in the quantities used had no favorable influence on the calcium assimilation of the liberally milking cow. We have attributed this to the failure of this species to absorb the oil. It occurred to us that probably if vitamin D

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were ingested in greater amounts and in a dry form, as for example, irradiated yeast, that better utilization of calcium might be brought about. Consequently, we planned our experiment with this point in view.

It is known that yeast (4) can be activated when exposed to the emanations of a quartz mercury vapor lamp and unpublished data show that it can be activated to a point where the daily ingestion of 200 gm. of irradiated yeast would be approximately equivalent to the ingestion of 4000 gm. of potent cod liver oil.

In planning this experiment we chose cows in the early part of their lactation because that is the period when it is most difficult to maintain them in positive calcium balance.

EXPERIMENTAL.

For this work three grade Holstein cows milking from 40 to 50 pounds of milk per day were selected. Cow 1 was fresh September 15, Cow 2 was fresh September 20, and Cow 3 was fresh September 15, 1928. The metabolism trial was begun on October 23, 1928, giving a lactating period of from 4 to 6 weeks before the actual initiation of the experiment. All of these cows were middle aged, having had from three to four calves, and were quiet and contented throughout the experiment. We mention this fact because at times, with young nervous animals, calcium assimilation may be distinctly disturbed. None of the animals was bred during the progress of the experiment. At the initiation of the experiment Cow 1 weighed 1189 pounds, and at the termination, 1171 pounds. Cow 2, at the beginning weighed 1138, and at the end 1122 pounds. Cow 3 weighed at the beginning of the experiment 1062, and 1058 pounds at the end.

The metabolism work was carried on for 8 weeks. During the first 4 weeks the cows received the regular basal ration plus 200 gm. daily of non-irradiated yeast, while in the second period of 4 weeks, they received the same basal ration plus a daily addition of 200 gm. of irradiated yeast. The daily ration consisted of 10 pounds of alfalfa hay, 25 pounds of corn silage, and 12 to 15 pounds of a grain mixture consisting of 59 parts of yellow corn, 25 parts of wheat bran, 15 parts of linseed oil meal, and 1 part of salt. The alfalfa and silage were kept constant while the amount of grain fed varied with the amount of milk produced.

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The yeast used was a dried brewers' yeast kindly donated by the Fleischmann Company. As already stated this was given in amounts of 200 gm. per animal per day. The irradiation of the yeast was carried out by exposing the material to a quartz mercury vapor lamp at a distance of approximately 3 feet. It was irradiated in thin layers for a period of 2 hours and in portions of 2 kilos; the yeast was raked over every half hour to insure more complete exposure. Actual tests of the potency of this yeast in its vitamin D content were carried out with rats by the usual procedure followed in this laboratory, and showed that as low an ingestion as 0.5 mg. per day for 10 days produced good calcification. The details of these tests will be presented elsewhere.

The alfalfa hay used in this experiment was a mixture of Colorado Hay 1 and Colorado Hay 2, both of which had been used in previous experiments (3) with cows on the question of the efficiency of hays cured under different conditions in maintaining calcium equilibrium. These hays had been cured in Colorado and shipped to this Station. Both contained some antirachitic properties, but neither of them fed in amounts of 10 pounds per day had shown themselves sufficiently antirachitic to maintain positive calcium balances in cows producing 40 to 50 pounds of milk per day. Throughout the entire yeast experiment of 8 weeks there was no interruption in the consumption of the ration by the three animals. In fact, there was no evidence of any disturbance that would mar the assurance of reliable data.

The calcium oxide intake per cow per week was approximately 825 gm., or 589 gm. of calcium. The phosphorus intake was approximately 450 gm. per cow per week. This gave us a calcium: phosphorus ratio of 1:0.76. This ratio may or may not be the optimum one for a liberally milking cow, but it is a ratio upon which we have sometimes seen positive calcium balances, and sometimes a negative balance in this species.

Records of the calcium and phosphorus balances are given in Tables I and II. It will be seen from these records that Cow 1 producing about 40 pounds of milk per day was in a positive balance in the non-irradiated yeast period by a total of approximately 9.44 gm. of calcium oxide, while in the irradiated yeast period a positive balance of 35.45 gm. was observed. There was no particular decline in the calcium of the feces under the influence of the

TABLE I.

Record of Calcium Balance and Milk Production of Cows 1, 2, and 3.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO excreted.	CaO intake.	Balance per wk.	Milk per wk.
			Cow 1. Non-irradiated yeast period.				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Oct. 23–29	549.19	186.60	19.48	755.30	824.42	+69.10	258.5
" 29–Nov. 5	651.46	194.16	12.20	857.82	827.6	−30.22	276.8
Nov. 5–12	621.78	199.81	12.94	834.53	831.23	−3.3	290.5
" 12–19	651.75	205.20	9.47	866.42	840.28	−26.14	291.6
			Irradiated yeast period.				
Nov. 19–26	584.86	213.56	13.07	811.49	840.28	+28.79	285.1
" 26–Dec. 3	608.01	219.59	8.72	836.32	840.28	+3.96	286.2
Dec. 3–10	601.42	199.65	16.10	817.17	840.28	+23.11	281.9
" 10–17	641.50	201.44	17.75	860.69	840.28	−20.41	279.2
			Cow 2. Non-irradiated yeast period.				
Oct. 23–29	562.46	253.37	12.00	827.80	838.57	+10.77	340.3
" 29–Nov. 5	581.43	261.67	9.59	852.69	845.22	−7.47	353.6
Nov. 5–12	566.35	265.59	10.36	842.3	848.87	+6.57	360.0
" 12–19	612.28	267.64	10.51	890.43	857.92	−32.51	353.0
			Irradiated yeast period.				
Nov. 19–26	599.88	248.13	10.5	858.51	857.92	−0.5	341.6
" 26–Dec. 3	616.17	261.17	14.11	891.45	857.92	−33.53	340.4
Dec. 3–10	597.31	257.78	14.24	869.33	857.92	−11.41	340.4
" 10–17	626.62	246.98	10.39	883.99	857.92	−26.07	337.9
			Cow 3. Non-irradiated yeast period.				
Oct. 23–29	559.20	196.42	12.95	768.57	817.58	+49.02	270.4
" 29–Nov. 5	546.06	184.55	8.08	738.69	827.61	+88.92	263.1
Nov. 5–12	642.02	189.56	7.27	838.85	831.23	−7.62	265.1
" 12–19	595.57	207.99	15.84	819.40	840.28	+20.88	282.8
			Irradiated yeast period.				
Nov. 19–26	569.18	203.13	15.49	787.80	840.28	+52.48	274.5
" 26–Dec. 3	588.43	198.85	20.58	807.86	840.28	+32.42	278.1
Dec. 3–10	593.77	202.48	26.05	822.30	840.28	+17.98	270.3
" 10–17	586.39	178.11	21.90	786.40	840.28	+53.88	245.2

TABLE II.

Record of Phosphorus Balance of Cows 1, 2, and 3.

Period.	P_2O_5 in feces.	P_2O_5 in urine.	P_2O_5 in milk.	Total P_2O_5 excreted.	P_2O_5 intake.	P_2O_5 balance.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Cow 1. Non-irradiated yeast period.						
Oct. 23–29	518.37	4.17	264.06	786.60	959.85	+173.25
" 30–Nov. 5	588.42	2.57	268.93	859.92	940.45	+80.53
Nov. 5–12	630.46	4.92	307.30	942.68	943.91	+1.23
" 12–19	625.66	5.76	309.78	941.20	952.56	+11.36
Irradiated yeast period.						
Nov. 19–26	609.29	5.35	278.29	892.93	952.56	+59.63
" 26–Dec. 3	567.86	4.71	324.84	906.41	952.56	+46.15
Dec. 3–10	539.34	4.33	285.40	829.07	952.56	+123.49
" 10–17	579.88	4.60	268.72	853.20	952.56	+99.36
Cow 2. Non-irradiated yeast period.						
Oct. 23–29	568.51	7.10	329.08	904.69	1076.08	+171.39
" 29–Nov. 5	668.96	3.20	308.23	980.39	1092.98	+112.59
Nov. 5–12	643.30	6.18	341.59	991.07	1096.44	+105.37
" 12–19	724.63	6.54	365.39	1096.56	1105.09	+8.53
Irradiated yeast period.						
Nov. 19–26	685.58	4.93	342.74	1033.25	1105.09	+71.84
" 26–Dec. 3	646.27	3.33	367.81	1017.41	1105.09	+87.68
Dec. 3–10	651.78	5.48	279.39	936.65	1105.09	+168.44
" 10–17	668.40	4.96	298.59	971.95	1105.09	+133.14
Cow 3. Non-irradiated yeast period.						
Oct. 23–29	542.42	4.75	243.07	790.24	901.74	+111.50
" 29–Nov. 5	543.18	3.68	241.28	788.14	940.45	+152.31
Nov. 5–12	623.23	6.26	246.73	876.22	943.91	+67.69
" 12–19	643.12	5.79	285.03	933.94	952.56	+18.62
Irradiated yeast period.						
Nov. 19–26	580.80	4.81	284.14	869.75	952.56	+82.81
" 26–Dec. 3	563.04	3.80	286.60	853.44	952.56	+99.12
Dec. 3–10	570.57	4.80	251.57	826.94	952.56	+125.62
" 10–17	562.85	4.18	239.34	806.37	952.56	+146.19

irradiated yeast. In the non-irradiated yeast period in the case of Cow 1, the amount of calcium oxide in the feces was 2474 gm., while in the irradiated yeast period the amount was 2435 gm. We emphasize this point particularly because in our work with goats it has always been observed that where we have changed an animal from a negative to a positive balance, which resulted in an effective and positive influence upon the calcium assimilation, the calcium content of the feces has been progressively decreased. With Cow 2, which gave approximately 50 pounds of milk per day, and was a better test of the influence of irradiated yeast than those cows that were lower producers, a negative calcium balance of 22 gm. prevailed during the non-irradiated yeast period, while a negative balance of 71 gm. prevailed during the irradiated yeast period. Likewise, with this animal there was no decrease in the calcium excretion in the feces as we changed from the non-irradiated yeast to the irradiated yeast. In fact, the amount of calcium excreted actually increased, being a total of 2322 gm. in the non-irradiated yeast period and 2419 gm. in the irradiated yeast period. Cow 3, which was milking about 35 pounds of milk per day showed a positive balance of 151 gm. of calcium oxide during the non-irradiated yeast period and a positive balance of 156 gm. during the irradiated yeast period. Calcium oxide excreted in the feces during the non-irradiated yeast period amounted to 2342 while the amount was 2337 gm. during the irradiated yeast period. This is a good illustration of how the volume of milk produced may influence calcium and phosphorus equilibrium. On the somewhat lower level of milk production, Cows 1 and 3 were able to maintain a calcium equilibrium on this ration while the other animal, Cow 2, milking somewhat higher, was unable to maintain calcium equilibrium on the same intake of calcium.

In the case of the phosphorus balances all of the animals in both the non-irradiated yeast period and the irradiated yeast period were positive. This condition has been observed before, and has convinced us that it is not difficult to maintain positive phosphorus balances in liberally milking cows at any stage of lactation if the level of phosphorus intake from grains and grain by-products is sufficiently high.

A most significant result from this experiment was the decided enrichment of the milk in vitamin D during the feeding of the ir-

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radiated yeast. These results will form the basis for a separate publication and will not be discussed here. Their bearing upon the calcium metabolism is, however, of great moment. The fact that the milk was enriched in vitamin D during the feeding of the irradiated yeast demonstrates conclusively that there was at least partial absorption of vitamin D into the blood. Yet with an apparent increase of vitamin D in the blood, the losses of lime still continued and presumably the draft was coming from the skeleton.

It is apparent from the experimental data that absorption of lime from the tract was not increased by the ingestion of more vitamin D, although part of the latter was absorbed, which would lead to postulation of the hypothesis that if conditions in the tract could be made more favorable for greater absorption of lime, the chances for maintaining calcium equilibrium would be better. In other words, the data indicate that in this species vitamin D and calcium level are not the only factors involved in calcium assimilation. The very fact that low lime green plant tissues supplemented with a calcium salt (5, 6) change negative calcium balances to positive ones in liberally milking cows, indicates that other factors than vitamin D and calcium level are to be considered. Green plant tissue is not particularly potent in vitamin D as compared with irradiated yeast, and yet its ingestion in the case of this species is decidedly favorable to the maintenance of positive calcium balances. The leads established from this experiment look hopeful for further elucidation of this difficult problem.

While part of the vitamin D of the irradiated yeast was absorbed, it is evident that not all of it left the intestinal tract. This is evident from the data secured through the extraction of the dried feces with ether to determine whether the feces still contained vitamin D. Portions of the dried feces were extracted for 48 hours with ether. The evaporated ether extract was saponified for 30 minutes with an equal volume of 20 per cent alcoholic potash. The saponification mixture was diluted with water and then extracted three times with ether. This procedure was followed in order to remove distasteful and possibly harmful substances from the first ether extract of the feces. After saponification the ether extract of the soaps was clear and straw-colored instead of black as was the original ether extract. The ration was

not unpalatable when the ether extract of the feces treated as described above was evaporated on it.

To test the antirachitic properties of these ether extracts, baby chicks were used with a basal ration of 97 parts of yellow corn, 2 parts of calcium carbonate, 1 part of salt, plus skimmed milk *ad libitum*. The amount of ether extract used with the rachitic ration represented a level of approximately 2 per cent of irradiated yeast in the mash. Five groups of chicks were used. The rations for the respective groups were: (1) basal ration plus 10 minutes ultra-violet light daily; (2) basal ration plus ether extract

TABLE III.

Vitamin D Content of Feces of Cows Fed Irradiated Yeast as Tested with Chicks.

Ration.	Silver nitrate test.	Ash of tibia.	Average weight at 6 wks.
		per cent	*gm.*
Basal plus irradiation (average six birds).....	++	42.30	262
Basal plus ether extract of feces of Cow 1 (average four birds).....	++	37.90	250
Basal plus ether extract of feces of Cow 2 (average four birds).....	++	40.50	225
Basal plus ether extract of feces of Cow 3 (average four birds).....	++	38.15	248
Basal (average six birds).....	−	30.6	154

++ indicates wide calcified metaphysis. − indicates wide non-calcified metaphysis.

of Cow 1; (3) basal ration plus ether extract of Cow 2; (4) basal ration plus ether extract of Cow 3; (5) basal ration only. The record of the results secured is given in Table III. These data show that there still remained in the feces some vitamin D. It would be impossible to decide at the present time whether the vitamin D found in the feces represented unabsorbed vitamin or reexcreted vitamin. It should further be emphasized that it is not claimed that ether makes a quantitative extraction of vitamin D from the feces.

The influence of irradiated yeast on the calcium and inorganic phosphorus content of the blood was also investigated. After 3 weeks of non-irradiated yeast feeding, blood was taken from each

animal and the calcium and inorganic phosphorus determined. The calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method and the inorganic phosphorus by the Briggs modification of the Bell-Doisy method. Also after 3 weeks of feeding irradiated yeast, samples of blood were again taken for

TABLE IV.

Total Calcium and Inorganic Phosphorus in Blood of Three Cows.

Cow No.	Non-irradiated yeast period.		Irradiated yeast period.	
	Ca per 100 cc. serum.	Inorganic P per 100 cc. serum.	Ca per 100 cc. serum.	Inorganic P per 100 cc. serum.
	mg.	*mg.*	*mg.*	*mg.*
1	11.3	4.7	11.3	6.0
2	10.1	5.7	11.2	6.3
3	11.5	6.2	11.8	5.9

TABLE V.

Per Cent of Calcium and Phosphorus in Milk.

Cow No.	Period.	Wk.	CaO	P_2O_5	Period.	Wk.	CaO	P_2O_5
			per cent	*per cent*			*per cent*	*per cent*
1	1. Non-irradiated yeast.	1	0.159	0.225	2. Irradiated yeast.	5	0.165	0.216
		2	0.154	0.214		6	0.162	0.250
		3	0.150	0.231		7	0.156	0.224
		4	0.155	0.230		8	0.156	0.212
2	1. Non-irradiated yeast.	1	0.164	0.213	2. Irradiated yeast.	5	0.161	0.220
		2	0.163	0.192		6	0.170	0.240
		3	0.160	0.209		7	0.165	0.180
		4	0.166	0.240		8	0.161	0.215
3	1. Non-irradiated yeast.	1	0.160	0.198	2. Irradiated yeast.	5	0.163	0.227
		2	0.154	0.202		6	0.157	0.227
		3	0.157	0.205		7	0.165	0.205
		4	0.161	0.254		8	0.160	0.215

calcium and inorganic phosphorus determinations. The data are shown in Table IV. The results secured show that these blood constituents were at normal levels in the non-irradiated yeast period and were not changed by the feeding of irradiated yeast.

Further there was no increase in the calcium and phosphorus content of the milk through the feeding of irradiated yeast. The

per cent of these two elements remained strikingly constant in the milk during the entire metabolism period. In support of this conclusion data covering the weekly analyses of the milk from the non-irradiated yeast period and the weekly analyses of the milk from the irradiated yeast period are given in Table V. Application of the sensitive alcohol coagulation test to the milk of both periods showed no differences in the coagulabilities. This sensitive test probably is related to the salt balance in the milk and had there been a change in the proportion of calcium and phosphorus in the milk, due to feeding of irradiated yeast, a coagulation with alcohol would have resulted. None of the milk from either period gave positive alcohol coagulation tests.

After finishing the metabolism experiment on December 16, 1928, the three cows were continued on irradiated yeast (200 gm. daily) in order to determine whether there might result any effect on the volume of milk secreted, and whether any other physiological disturbances might present themselves. They received irradiated yeast for a period of approximately 8 months. The estrous cycles were regular in Cows 1 and 3. In Cow 2 the estrous cycle was less regular. The record of these cycles was only a matter of gross observation, and only had they ceased could we have suspected any deleterious influence from the irradiated yeast in the period of observation. The production of milk has been well sustained with these animals. It has certainly not fallen below the curve of expectation. In October, 1928, Cow 1 was producing 40 pounds of milk daily; in June, 1929, 30 pounds daily. With allowance for a monthly decrease of 6 per cent (an average figure often used), the expected milk production should have been but 25.9 pounds. Cow 2 in October produced 48 pounds daily; in June, 1929, 36 pounds; calculated expectation, 31.1. Cow 3 in October produced 40 pounds of milk daily; in June, 1929, 32 pounds; calculated expectation, 25.9. Certainly from these data, although too meager for broad generalization, there is no indication of a disturbing action of this amount of irradiated yeast on milk production.

SUMMARY.

1. Irradiated yeast, potent in vitamin D, showed no positive influence in improving the lime assimilation of liberally milking

cows, receiving a ration of alfalfa hay, corn silage, grains, and grain by-products, although vitamin D was absorbed into the blood as evidenced by the enrichment of the milk in this factor.

2. The composition of the blood in respect to calcium and inorganic phosphorus was not changed during the irradiated yeast-feeding period.

3. The per cent of calcium and phosphorus in the milk was not changed by the feeding of irradiated yeast.

4. Milk production was well sustained during the 8 months of irradiated yeast feeding, and there was no indication of disturbed physiological functioning during this period.

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THE COMPOSITION OF NORMAL RAT BLOOD.*

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(Received for publication, November 27, 1929.)

A review of the literature on the composition of rat blood shows that very little work has been done in this field. In 1913 Folin and Morris (2) published data on the uric acid, non-protein nitrogen, and urea content of rat blood. In their work they used the mixed blood of six normal rats and found 2 mg. of uric acid, 38 mg. of non-protein nitrogen, and 22 mg. of urea per 100 cc. of blood. The urea value expressed as urea nitrogen would be 10.25 mg. Voegtlin, Dunn, and Thompson (5), in 1924, found an average of 113 mg. of sugar per 100 cc. of blood.

In this paper we present the data obtained from the analysis of the blood from twenty-eight normal and three abnormal rats. Because of the small quantity of blood obtainable from a rat, it was impossible to make a complete analysis of the blood of any one rat, so the animals were divided into two groups. With Group I, sugar, non-protein nitrogen, creatinine, and creatine plus creatinine were determined. With Group II urea nitrogen, uric acid, and sodium chloride were determined.

In all the work here reported blood from individual rats was analyzed. To obtain the blood samples the animals were decapitated, care being taken to excite the animals as little as possible. Lithium oxalate was used to prevent clotting. Each animal was later autopsied to note any evidences of disease. The methods used for the determination of sugar, non-protein nitrogen, creatinine, and creatine plus creatinine were those of Folin and Wu (3, 4). Because of the high non-protein nitrogen content, 5 cc.

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TABLE I.

Composition of Rat Blood. Group I.

The composition is expressed in mg. per 100 cc. of blood.

Rat No.	Sex.	Breed.	Age.	Sugar.	Non-protein N.	Creatinine.	Creatine plus creatinine expressed as creatinine.
			mos.				
361	Male.	Pied.	3	125.3	45.2	1.24	6.51
364	"	"	3	132.3	40.0	1.34	6.57
349	Female.	"	4	123.2	44.8	1.32	6.57
336	Male.	Albino.	5	123.1	47.8	1.34	6.52
Average of four rats 3 to 5 mos. old.....				126.0	44.5	1.31	6.54
	Male.	Pied.	6	128.2	45.7	1.23	6.62
	"	"	6	127.2	43.7	1.27	6.54
	"	"	6	130.1	47.8	1.23	6.57
	"	Albino.	6	118.0	47.3	1.24	6.64
	"	"	6	116.7	49.1	1.32	6.54
310	"	"	8	126.6	38.6	1.34	6.12
266	"	Pied.	9	112.3	46.5	1.36	6.52
294	"	"	9	124.2	43.2	1.34	6.41
282	"	"	10	124.2	47.8	1.34	6.54
281	"	"	10	110.1	46.5	1.28	6.98
Average of ten rats 6 to 10 mos. old.....				121.8	45.6	1.30	6.55
131	Male.	Pied.	13	124.6	43.4	1.22	6.42
145	Female.	"	14	115.2	45.6	1.23	6.12
118	"	"	16	116.7	45.2	1.15	6.00
Average of three rats 13 to 16 mos. old..				118.8	44.6	1.20	6.18
301	Female.	Pied.	7	435.2	55.8	1.42	6.42
191	"	"	12	153.6	49.4	1.24	6.60
146	"	"	12	185.9	58.0	1.28	6.66
Average of three rats with bad lungs.....				261.6	54.4	1.31	6.56
Average of seventeen normal rats.....				122.2	45.2	1.28	6.48
" " fourteen " males....				123.1	45.2	1.29	6.54
" " three normal females.....				118.4	45.1	1.23	6.23
" " four albino rats.....				121.1	45.7	1.30	6.46
" " thirteen pied "				122.6	45.0	1.27	6.41

of the ammonium sulfate standard solution were used instead of the 3 cc. usually recommended. In the determination of urea nitrogen an aeration-Nesslerization method was employed, 5 cc. of the Folin-Wu filtrate being used for analysis. Uric acid was determined by Benedict's (1) direct method and chlorides by the method of Whitehorn (6).

Table I summarizes the data collected with Group I. The results are arranged in order according to the age of the animals and averages are given for the different age groups, for male and

TABLE II.

Composition of Rat Blood. Group II.

The composition is expressed in mg. per 100 cc. of blood.

Rat No.	Sex.	Breed.	Age.	Urea N.	Uric acid.	NaCl
			mos.			
4277	Male.	Pied.	3	18.3		
668	"	Albino.	7	16.5	1.39	477.0
	"	Pied.	7	16.3		
	"	"	7	14.3	1.35	502.8
	"	"	7	14.5		
	"	"	7	9.9		
627	"	"	8	15.2	1.92	472.8
599	Female.	"	9	20.6	2.12	550.7
600	Male.	"	9	14.2	2.12	524.0
555	"	"	13	15.2	1.69	507.0
507	Female.	Albino.	16	16.2	2.40	572.0
Average.....				15.6	1.86	515.2

female, and for albino and pied rats. Table II records the results obtained with Group II. Because of the small number of individuals, no attempt has been made to divide them into groups.

An inspection of Tables I and II shows that in most respects rat blood is quite similar in composition to human blood. The most noticeable exception is in the case of non-protein nitrogen where the average value is considerably above that for human blood. Blood sugar, urea nitrogen, and chloride values are slightly higher than one finds in normal human blood. In connection with the values given for uric acid it should be noted that these possibly do not represent true values for this constituent since the

method used is said by the author to be applicable only to human blood. Whether or not this method is applicable to rat blood remains to be determined.

From Table I, where average values are given for various age groups, male and female, and albino and pied rats, it is evident that there is very little variation in the various groups. Blood sugar values decrease slightly with age and females give a lower average than males.

On autopsy, three rats in Group I were found to have badly infected lungs. Since the blood from these animals showed marked deviations from the normal, with respect to sugar and non-protein nitrogen values, the results are included. The average blood sugar value for this group is over twice that for normal animals. Non-protein nitrogen values are definitely higher than the normal.

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AN INHERITANCE STUDY OF THE DISTRIBUTION OF VITAMIN A IN MAIZE.

II. VITAMIN A IN HYBRID RED MAIZE.*

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(Received for publication, December 30, 1929.)

The knowledge that in hybrid corn there are strains which give increased yields and also possess other desirable qualities (1) has resulted in increased production of these types of corn until they have assumed an economic importance. Not only are hybrids produced by crossing yellow and white corn but also by crossing the red varieties with other strains of corn. The quantities of these hybrids which are available for feeding purposes make it desirable to secure information as to the inheritance of vitamin A in relation to color characteristics of the pericarp and endosperm.

Hauge and Trost (2) made an inheritance study of the distribution of vitamin A in F_2 segregating kernels of crosses of yellow dent and white dent corn. They found that vitamin A is present only in kernels possessing yellow endosperm. The corn used in their experiments possessed colorless pericarp. In order to study the possible effect of pericarp color, these inheritance studies were extended to include strains of corn possessing red pericarp.

EXPERIMENTAL.

The lots of corn used in these experiments were selected from two hybrid strains, one of which possessed colorless pericarp and the other deep red pericarp. From the ears of corn of the F_2 generation of the cross white dent by yellow dent, equal quantities of first, F_2 yellow, YYY, and second, F_2 white, yyy, were

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selected from each ear. From the ears possessing red pericarp, third, red grains with yellow endosperm, and fourth, red grains with white endosperm were secured from the same ears. These were identified by removing a portion of the pericarp from each kernel to determine the color of the endosperm and then separating them into the two respective classes. These four classes constituted the material to be tested for vitamin A.

In testing the various lots of corn for vitamin A, the ophthalmic reaction was used as the main criterion for the presence or absence of vitamin A. Young albino rats were employed as the experi-

TABLE I.

Composition of Rations Used to Test Various Types of Corn for Vitamin A.

Lot No.	1	2	3 and 5	4 and 6
	per cent	*per cent*	*per cent*	*per cent*
F_2 yellow, YYY	20			
F_2 white, yyy		20		
Red grains, yellow endosperm			20	
" " white "				20
Casein	15	15	15	15
Lard	5	5	5	5
Dextrin	52	52	52	52
Salt Mixture 185.*	3	3	3	3
Yeast	3	3	3	3
Agar-agar	2	2	2	2

All animals were protected against rickets by irradiation with ultra-violet light.

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

mental animals. The rats used in these experiments were selected from litters reared from a group of the stock colony which had received a low vitamin A diet. Litter mates were distributed through the various lots. The rats were confined in individual cages provided with false screen bottoms to prevent access to excreta. The food was given *ad libitum* in the McCollum type feeding cups. The composition of the rations is given in Table I. All animals were protected against rickets by irradiation with ultra-violet light. Weekly records of weights of the animals and feed consumption were kept.

The results of these tests are given in Chart 1. It is seen that

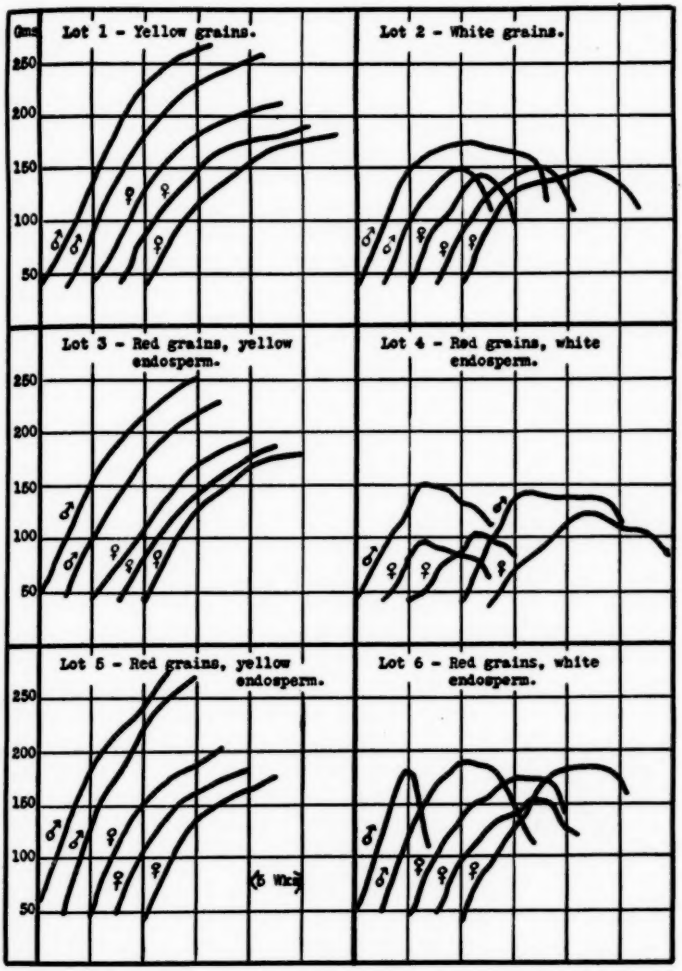

CHART 1. The results of an inheritance study of vitamin A in hybrid corn possessing colorless pericarp (Lots 1 and 2) and red pericarp (first test, Lots 3 and 4; second test, Lots 5 and 6). Vitamin A was found to be present only in the kernels with yellow endosperm, and lacking in the kernels with white endosperm, even when the latter were grown on the same ears as those with yellow endosperm.

vitamin A is present only in the kernels possessing yellow endosperm, while the kernels with white endosperm were lacking in vitamin A, even though they were grown on the same ear as those with the yellow endosperm. The rations of Lot 1, containing yellow corn and Lots 3 and 5 containing red grains with yellow endosperm, proved equally effective in promoting growth and preventing ophthalmia.

DISCUSSION.

In 1920, Steenbock and Boutwell (3) showed that King Phillip's Flint which is a red corn with yellow endosperm contained vitamin A, while Cardinal King which had a white endosperm, did not contain a demonstrable amount of the vitamin. These results were similar to those obtained with white and yellow dent corn. In studying variegated maize where there was an intermingling of color characteristics they separated the kernels into two classes, one with predominating yellow kernels and the other with a mixture of kernels with pale yellow and white endosperms. Their results showed that although growth was not satisfactory, due to the lack of the antirachitic factor (4), no ophthalmia occurred. These experiments might have shown a close relationship to the present data had they made their separation completely; *i.e.*, if instead of contaminating the white kernels with the pale yellow, they had separated them into one group containing yellow endosperm and the other pure white endosperm. Ophthalmia would then undoubtedly have occurred in the rats fed the kernels of white endosperm.

The maizes used in our experiments were different from those used by Steenbock and Boutwell (3). They used varieties of red corn with either white or yellow endosperm while in these experiments corn from a hybrid strain possessing homozygous white, homozygous yellow, and heterozygous yellow endosperm on the same ears was used. From the same ears, whether the pericarp was red or colorless, grains of yellow endosperm and of white endosperm were separated.

At the levels of corn (20 per cent) used in these experiments it was not possible to detect any differences in the vitamin content of the yellow grains with red or colorless pericarp, nor was there any indication that vitamin A was transferred to the kernels of

white endosperm. These experiments with the red grains further substantiate the previous work (2) that there is a close physiological association between vitamin A and yellow endosperm character.

SUMMARY.

1. In hybrid red maize, vitamin A is associated with yellow endosperm and is lacking in the corn grains with pure white endosperm, even when grown on the same ears as those possessing grains with yellow endosperm.

2. The color of the pericarp appears to have no effect on the vitamin A content of corn.

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AN INHERITANCE STUDY OF THE DISTRIBUTION OF VITAMIN A IN MAIZE.

III. VITAMIN A CONTENT IN RELATION TO YELLOW ENDOSPERM.*

By SIGFRED M. HAUGE and JOHN F. TROST.

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In a previous publication (1) dealing with an inheritance study of the distribution of vitamin A in F_2 segregating kernels of crosses of yellow dent and white dent corn, it was found that vitamin A is always associated with the yellow endosperm and is lacking in the grains with white endosperm, even when these are grown on the same ears as the yellow grains. This association was further confirmed (2) when these studies were extended to hybrid red maize in which vitamin A was found to be present only in the kernels possessing yellow endosperm. It was further shown that the color of the pericarp was without effect in the formation of vitamin A in the kernel.

These results indicated the existence of a close physiological relationship between the yellow color character and the formation of vitamin A. Since at the levels of corn used in these experiments, the three classes possessing yellow endosperm, heterozygous F_2, homozygous F_2, and homozygous parent, were equally effective in preventing ophthalmia, two explanations for this association of yellow endosperm with vitamin A formation may be possible. If the vitamin A content of all types of yellow corn with various degrees of color intensity is the same, this would suggest the possibility of a catalytic action by the yellow pigments in the formation of vitamin A, while if the vitamin A content

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parallels the amount of color character, either the physiological relationship must be very close or the factor acting as vitamin A may be the yellow pigment itself (3, 4).

To test this relationship, it was desirable to compare maizes possessing as wide a range of yellow endosperm character as possible. This was done by producing two classes of grains possessing respectively the following genetical constitution, Yyy and YYY.

The material used in this study consisted of open pollinated ears of Reid Yellow Dent and of ears borne on Johnson County White Dent hybrid plants which had grown adjacent to a number of strains of Reid Yellow Dent corn. These white and yellow strains pollinated at approximately the same time. Under these conditions, ears were obtained from the Johnson County White Dent plants which bore a liberal percentage of white capped grains with yellow endosperm. Such grains were formed as a result of natural cross pollination with pollen from the Reid Yellow Dent plants and represented the lowest possible genetical contribution to the formation of yellow endosperm where a single pair of genetic factors controls these color relations between white and yellow dent. On the other hand, the pure yellow grains borne on the Reid Yellow Dent ears represented the full complement of factors for the development of yellow endosperm from both the male (pollen) and female (silk) parents. The genetic constitution of the endosperm of these two samples is represented as follows: F_1 Johnson County White Dent × Reid Yellow Dent gave Yyy, two white factors from the female and one from the male; Reid Yellow Dent gave YYY, yellow factors from both parents.

Only crossed grains were selected from 150 representative ears of the Johnson County White Dent and made into one composite. A similar sample composed only of grains with pure yellow endosperm was drawn from 150 ears of Reid Yellow Dent.

EXPERIMENTAL.

For the determination of the vitamin A content of these two samples of corn, YYY and Yyy, the curative test was used for all lots except one for which the prophylactic test was used as confirmation. During the preliminary depletion period, the rats were restricted to the vitamin A-deficient diet until the storage of

vitamin A in the animals was exhausted as evidenced by definite symptoms of vitamin A deficiency. At this point, usually after 3 to 4 weeks, the animals were transferred to the respective test rations.

The general plan of selection of rats, caging, and management was used as previously described (2). The composition of the rations is given in Table I. Antirachitic properties were imparted to the rations by irradiation with ultra-violet light. Since it had been shown previously (1) that 20 per cent of the various

TABLE I.

Composition of Rations Used to Test Vitamin A Content of Corn.

Lot No.	1	2	3	4	5	6	7	8	Vitamin A-deficient diet.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Yellow endosperm, YYY					3	5	7	10	
Hybrid, white × yellow endosperm, Yyy	5	10	15	20					
Dextrin	58	53	47	43	60	58	56	53	63
Casein	18	18	18	18	18	18	18	18	18
Yeast	3	3	3	3	3	3	3	3	3
Salt Mixture 185.*	4	4	4	4	4	4	4	4	4
Agar-agar	2	2	2	2	2	2	2	2	2
Lard	10	10	10	10	10	10	10	10	10

Antirachitic properties were imparted to all rations by irradiation with ultra-violet light.

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

genetical groups of corn prevented ophthalmia with no apparent differences in their ability to promote growth, this level was taken as the upper dose limit. The percentage level of corn was decreased until the amount of corn was inadequate to meet the vitamin A requirements of the animals and nutritional failure resulted. With the Yyy corn, levels of 20, 15, 10, and 5 per cent were tested and 10, 7, 5, and 3 per cent of YYY were used.

The results of these tests are given in Chart 1. It is apparent that the critical level for the crossed seed with a single dosage of the genetical factor for yellow endosperm, Yyy, is approximately

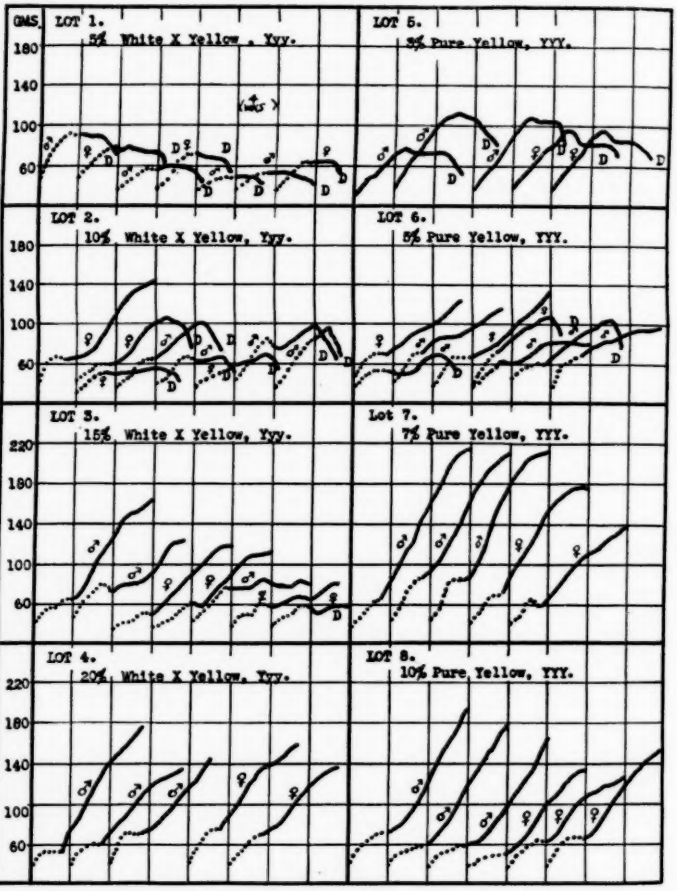

CHART 1. The responses of rats in quantitative comparisons of the vitamin A content of dent corn having pure yellow endosperm, YYY, with similar corn having yellow endosperm of the genetic constitution Yyy.

15 per cent, while that of the pure yellow endosperm, YYY, corn is about 5 per cent. Lot 5, the prophylactic test being used, shows that 3 per cent is below the critical level. 7 per cent of the YYY corn gave good growth while it took 20 per cent of the Yyy corn to give comparable results.

DISCUSSION.

It is evident from these results that the mere presence of yellow pigments in yellow corn does not insure the maximum formation of vitamin A. Since Lot 2 receiving 10 per cent of Yyy corn gave markedly different responses from Lot 8 receiving an equivalent amount of YYY corn, it is apparent that the carotinoids in corn do not function merely as catalytic agents in the formation of vitamin A.

In a series of experiments with each type of corn, tests have been made both below the critical levels of vitamin A as evidenced by nutritional failure and above this level as shown by the cure of ophthalmia and resumption of growth. These results place the critical level of the pure yellow corn as a source of vitamin A at about 5 per cent of the rations and that of the crossed grains at 15 per cent. Those animals which received 7 per cent of pure yellow corn recovered from ophthalmia and made about the same gain as those that received 20 per cent of the crossed grains. Thus, the results obtained both at the critical level and above indicate that the vitamin A content of pure yellow corn of the genotype YYY is approximately 3 times that of the crossed grains of the genotype Yyy. This would indicate that the ratio of vitamin A content in YYY and Yyy corn is about 3:1. This is the same ratio as that borne by the genetic factors governing yellow endosperm formation in these two classes of corn.

The quantitative differences in the vitamin A content of these two classes of corn, together with the entire absence of this vitamin in corn with pure white endosperm of the genotype yyy, make it apparent that the biochemical character, vitamin A, is controlled by ordinary genetic factors. As vitamin A has never been found to be associated with white endosperm in dent corn either in our own experiments or in those reported by others, it appears that vitamin A and yellow endosperm formation are determined by the same genes.

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SUMMARY.

1. Comparisons were made of the vitamin A content of two classes of dent corn, YYY and Yyy, possessing as wide a range of yellow endosperm character as possible.

2. The results show that the critical level for the Yyy corn is approximately 15 per cent, while that of the YYY corn is about 5 per cent.

3. 7 per cent of YYY corn gave good growth while it took 20 per cent of Yyy corn to give comparable results.

4. The vitamin A content of dent corn is controlled by ordinary hereditary factors. These genes are the same as those governing development of the yellow endosperm.

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UNLAKED BLOOD AS A BASIS FOR BLOOD ANALYSIS.

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Almost every method included in the Folin-Wu system of blood analysis has been repeatedly revised and amended in the many papers on blood analysis which have appeared in this *Journal* since the publication of that system in 1919. But the starting point for analyses, the preparation of the protein-free filtrate, has been accepted by all, and it is still used practically just as recommended in the original paper. Some of the proposed revisions of the Folin-Wu system, such as the use of heat and cyanide for the uric acid determination (Benedict) have undoubtedly been very important, but the multiplicity of revisions and modifications have also produced much confusion among those who only want to apply the methods. It is not without regrets, therefore, that I in this paper shall propose a radical revision of the Folin-Wu method for preparing protein-free blood extracts.

It has become increasingly evident in the last few years that most of the uncertainties inherent in the methods which have been most frequently the subject of debate and revisions owe their uncertainty to the fact that the blood filtrates include constitutents of the disintegrated blood corpuscles. Most of us probably have felt for years that it would be more satisfactory for nearly every purpose if the analyses could be based on blood plasma rather than on whole blood. Wu (1) suggested a long time ago that this should be done. But in general, especially for clinical purposes and for work on small animals, such a proposal is quite impractical. On the other hand, it is eminently practical to prepare from whole blood aqueous filtrates or extracts which contain the plasma constituents together with the readily diffusible products of the blood cells, but which are free from the disintegration products of the cells.

This new form of blood extract was developed in connection with a study of the puzzling problem as to why one cannot recover known quantities of uric acid when these are added to blood which is essentially free from uric acid.

It has been taken for granted that the large losses (20 to 40 per cent) encountered when uric acid is added to sheep blood must be due to adsorption of uric acid by the protein precipitates. Losses due to such adsorption are, however, practically negligible. The large deficits encountered can be traced to the fact that something in the blood filtrates depresses the color reaction upon which the determination is based. The interfering products in whole blood extracts occur together with the non-uric acid products which themselves give a color with the uric acid reagent. The latter, as shown by Benedict (2), as well as by Hunter and Eagles (3), come practically exclusively from the disrupted cells.

In order to demonstrate the disturbing effects of the blood cell residues present in the regular Folin-Wu filtrates it seems best to describe briefly two or three crucial experiments.

For these experiments, the following solutions are required.

1. The regular Folin-Wu sodium tungstate solution (10 per cent) and $\frac{2}{3}$ N sulfuric acid.
2. A solution containing 12 gm. of sodium tungstate and 30 gm. of anhydrous sodium sulfate per liter.
3. $\frac{1}{3}$ N sulfuric acid.
4. Sheep blood.
5. A standard uric acid solution containing 5 mg. in 200 cc.
6. Uric acid reagent and sodium cyanide solution. The uric acid reagent should be free from phenol reagent. In my experiments, the uric acid reagent and the new cyanide solution described in the following paper were used.

Experiment I.—To a 100 cc. flask add, *in the order named*, 20 cc. of water, 20 cc. of the sulfate-tungstate solution, and 5 cc. of sheep blood. The blood, of course, is not laked in this hypertonic mixture. Let stand for 5 minutes, with occasional very gentle shaking. Add slowly, with gentle shaking, 5 cc. of $\frac{1}{3}$ N sulfuric acid. Centrifuge in 15 cc. tubes.

Next prepare an ordinary Folin-Wu filtrate from another 5 cc. of the same blood as follows: lake the blood (5 cc.) with 35 cc. of

water, add 5 cc. of sodium tungstate and finally add, with shaking, 5 cc. of $\frac{2}{3}$ N sulfuric acid, and filter.

Determine in a comparative way the color which each of these two blood filtrates gives when treated in the ordinary way as for a direct determination of uric acid. The blood filtrate prepared in the new way should give just a trace more color than is obtained when water is substituted for blood filtrate. The regular Folin-Wu filtrate, on the other hand, will give a color corresponding to from 0.5 to over 1 mg. per cent of uric acid.

Next add 1 cc. of the uric acid solution (*a*) to 5 cc. of water, (*b*) to 5 cc. of the unlaked blood filtrate, and (*c*) to 5 cc. of the regular filtrate; and make a direct uric acid determination. Using (*a*) as the standard, set at 20 mm. for the color comparison, (*b*) will read a fraction above or below 21 mm.; (*c*) on the other hand will read somewhere between 25 and 30 mm., although in the preliminary test it gave a blank equivalent to 0.8 mg. per cent or 16 per cent of the amount of uric acid involved in this experiment.

Experiment II.—Repeat the preparation of the two blood filtrates exactly as described in Experiment I, except that in each case 10 cc. of the standard uric acid solution are substituted for 10 cc. of water.

Determine the uric acid by the direct process in each filtrate, using 1 cc. of the uric acid solution plus 4 cc. of water as a standard. The colorimetric reading from each filtrate will be a practically exact duplicate of the corresponding reading obtained in the second part of Experiment I.

In Experiments I and II, the added uric acid is equivalent to 5 mg. per cent, but exactly the same sort of results are obtained if the concentration of the uric acid solution is doubled so that the additions become equivalent to 10 mg. per cent.

The significance of these results is clear.

1. There is no material loss of uric acid through adsorption by the protein precipitate.

2. The disappearance of uric acid is due to some inhibiting action by the blood filtrate on the color development.

3. Both the inhibiting action and the blank due to reactive non-uric acid substances in blood are almost completely eliminated by the new method for removing the blood proteins.

The disturbing materials encountered when working with

laked sheep blood are no less evident when one works with bloods which contain preformed uric acid, such as human blood. Evidence on this point is given in the tabulated analyses recorded at the end of the following paper. The current direct methods for the determination of uric acid in human blood involve negative errors of 25 per cent or more, and fluctuating positive errors of unknown magnitude.

For the new form of protein precipitation, one does not work exactly as in the experiments described above. The tungstate-sulfate solution used in those experiments was of double strength, so as to leave room for the addition of uric acid. For the ordinary preparation of unlaked blood extracts, the following solutions are employed.

1. A solution containing 15 gm. of anhydrous sodium sulfate and 6 gm. of sodium tungstate per liter.

2. $\frac{1}{3}$ N sulfuric acid.

Transfer 40 cc. (8 volumes) of the sulfate-tungstate solution to a small flask. With a 5 cc. pipette (or blood pipette), add 5 cc. of blood. Mix without any rough shaking, so as not to damage the cells mechanically, and let stand, with occasional very gentle shaking, for 5 minutes, or as much longer as may be convenient. With a pipette, add rather slowly, with constant but gentle mixing, 5 cc. (1 volume) of $\frac{1}{3}$ N sulfuric acid. Transfer the mixture to 15 cc. centrifuge tubes, and centrifuge for 10 minutes, at a moderate speed. The supernatant liquid should be perfectly colorless, and clear as water.

In this unusual form of blood protein precipitation, the blood cells are scarcely, if at all, acted upon by the tungstic acid. Only one-half as much sodium tungstate is used as in the Folin-Wu precipitation, and the added sulfuric acid is also one-half of the regular amount. One obtains, therefore, a mixture of precipitated plasma proteins and shrunken but not disintegrated blood cells. The blood cells cannot remain intact indefinitely in such a mixture, partly because the acidity is unavoidably somewhat greater than in the regular Folin-Wu filtrates. After about 45 minutes, if left standing, the cells begin to disintegrate. If one uses filtration instead of the centrifuge for separating the protein-free extract, one accordingly finds (*a*) that effective filtration stops in 20 to 30 minutes, because the blood cells clog the filter pores, and (*b*)

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that if left on the filter, the disintegrating blood cell material will, in time, begin to pass through. Centrifuging, rather than filtration, should be used. One practical advantage gained by this new process for precipitating the proteins is that water-clear extracts are so easily obtained by centrifuging, and the volume of extract secured is materially larger than that obtained in the Folin-Wu process.

I am well aware that the use of unlaked blood extracts as a basis for blood analysis may be open to certain theoretical objections, but at least equally strong objections can really be raised against the protein-free extracts from a mixture of plasma and disintegrated blood cells.

Over one-third of the total non-protein nitrogen in the Folin-Wu filtrates is represented by unknown products. This undetermined nitrogen comes very largely from the disintegrated blood cells. Most of this residual nitrogen, therefore, should fail to appear in the filtrates obtained from unlaked blood, and these filtrates should yield correspondingly lower values for the total non-protein nitrogen. New sets of figures, representing the normal range of variations for the different blood filtrate constituents must be determined on unlaked blood. Only a few figures for the uric acid and the total non-protein nitrogen are given now, at the end of the following paper. These comparative determinations were made by Dr. Andrea Svedberg.

The Folin-Wu method for the blood sugar determination is applicable to the new filtrate. In fact, by adding 2 per cent sodium sulfate to the sugar standard, to approximately equalize the salt concentration in the filtrate, and then using the method as recently described (4), it becomes more reliable than it has ever been. The non-sugar reducing material (glutathione and thioneine, Benedict (5)) is eliminated, and through the salt effect the proportionality has become just about perfect. The sugar values found are 10 to 15 mg. per cent lower than the values given by the Folin-Wu filtrate, and Dr. Svedberg has found that the non-fermentable "sugar" has practically vanished.

As yet, we have made only a few comparative urea determinations, but these indicate that the values obtained from the unlaked blood extracts are substantially identical with those obtained from the Folin-Wu filtrates.

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AN IMPROVED METHOD FOR THE DETERMINATION OF URIC ACID IN BLOOD.

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INTRODUCTION.

In the preceding paper is shown experimentally that it is impossible to say exactly what is being determined when the direct uric acid method is applied to Folin-Wu blood filtrates. By using the more laborious indirect method of precipitating with acid silver lactate and extracting with acid sodium chloride as worked out by Folin and Wu, more valid results are obtained. But the disturbing substances are scarcely *completely* separated from the uric acid by this process, and, besides, it is rather hopeless to ask clinical workers to go back to that distinctly more tedious process. My efforts to retain the direct method without sacrifice of accuracy led finally to the preparation of the blood filtrates from unlaked blood as described in the preceding paper. Quite apart from the uncertainties which come from using laked blood filtrates, the uric acid determination has been beset by a great many difficulties. Each reagent employed presented a separate problem. It took years to find a truly serviceable, indefinitely stable, standard solution of uric acid. It took much effort to prepare a uric acid reagent completely free from phenol reagent. The use of cyanide as the only alkali, brought in another set of difficulties, such as the deterioration and the different blanks. Finally, there was always the practical, serious difficulty of getting turbidities instead of crystal-clear solutions.

In the light of the history of past efforts in this field, it is perhaps too much to expect that all of these difficulties have been surmounted, but the method, as now revised, certainly seems good.

Preparation of the Reagents for Colorimetric Uric Acid Determinations.

1. Sodium Cyanide-Urea Solution.—Different brands of sodium cyanide differ widely (*a*) in the intensity of blue color which they give with the uric acid reagent (the blank), (*b*) in the intensity of color which they give with 0.02 mg. of uric acid and 1 cc. of the uric acid reagent, (*c*) in the rate with which different cyanide solutions deteriorate, and (*d*) in their tendency to give turbidities with blood filtrates. It seemed not particularly hopeful to try to secure a stable, uniform, standard cyanide solution in view of all these variables. Considerable progress toward this goal has been attained, however.

There is no longer any need to use sodium cyanides, the freshly prepared solutions of which give *any color at all before heating* with 1 cc. of uric acid reagent. Merck's best grade of coarsely *granular* sodium cyanide is good in this respect, but there are others. When a suitable sample has been found, it should be kept in a wide-mouthed glass-stoppered bottle, and it will then remain unchanged for years. I have one sample about 4 years old. The present investigation of the cyanides was begun primarily for the purpose of trying to circumvent their tendency to give turbidities with blood filtrates. Several years ago, it was found that lithium salts are very useful in reducing the tendency to give turbidities instead of crystal-clear solutions. This remedy was pushed to the utmost by incorporating much lithium salt in the uric acid reagent. The remedy was in fact pushed a little too far, because an excess of lithium salts also gives rise to some slight turbidity when heat is applied. Many probably have come to accept this slight turbidity as unavoidable. Since the amount of lithium employed should be diminished rather than increased, a search was begun for some other product which, like lithium salts, might have the effect of inhibiting the formation of insoluble phosphotungstates as produced in the uric acid determination. The desired result was obtained by a liberal use of urea. Other substances capable of inhibiting the turbidity formation probably could be found, but urea, when incorporated in the cyanide solution contributes another very important result. It stabilizes the cyanide. The spontaneous decomposition of sodium cyanide solutions with the formation of ammonia seems to be entirely prevented by the urea. This ammonia formation is responsible

for the greater tendency of old cyanide solutions to yield turbidities when clear solution is wanted and, in addition, it cuts down the amount of color obtainable from a given quantity of uric acid.

It was hoped that sodium cyanide solutions stabilized by means of urea would retain the full power of freshly prepared solutions in developing the maximum color with 0.02 mg. of uric acid, but this hope failed of fulfilment. Solutions kept for a couple of weeks at ordinary room temperatures gave distinctly less color than fresh ones.

As a matter of fact, the most important reason why solutions of different batches of sodium cyanide yield different depths of blue with 0.02 mg. of uric acid is their difference in carbonate content, and their deterioration in this respect with age is also due in part to the slight but continuous formation of more carbonates. This form of cyanide decomposition evidently is not prevented by the urea, but the process is certainly slow. Since the only effect is a moderate loss of color when old urea-cyanide solutions are used, this defect might perhaps be accepted. But since this new form of cyanide solution has such excellent keeping qualities, it seemed well worth while also to eliminate the disturbing effects of the carbonates.

The new cyanide solution for uric acid determinations is prepared as follows:

Transfer approximately 50 gm. of sodium cyanide to a 2 liter beaker. Add 700 cc. of water, and stir nearly continuously until substantially complete solution is obtained. Add 300 gm. of C.P. urea (Merck's) and stir; complete solution is obtained in a couple of minutes, but the solution is not clear, mostly because of impurities in the urea. Transfer the solution to a 2 liter flask, add a few gm. (5 or 6) of C.P. calcium oxide and shake moderately for 4 or 5 minutes.

The solution will now filter rapidly and perfectly clear through a Whatman No. 41 filter paper. The filtrate will be free from carbonates and will yield 15 to 30 per cent more color (with 0.02 mg. of uric acid) than before the removal of the carbonate. Unfortunately, the filtrate contains a surplus of calcium hydroxide which will form insoluble calcium phosphate with the uric acid reagent, hence the solution cannot be used as it is at this stage, unless one is willing to remove the calcium phosphate by centrifug-

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ing before making the color comparison. On the other hand, a little new carbonate is slowly forming, and it is, therefore, desirable to keep the solution in tightly stoppered bottles, and to leave in it the surplus calcium hydroxide. In laboratories where only a single occasional uric acid determination is made, the most practical procedure is probably to use the solution as it is, because the calcium phosphate formed during the reaction, unlike the insoluble phosphotungstates ordinarily encountered, is easily removed by centrifuging and yields crystal-clear supernatant solutions suitable for the color comparison. Where uric acid determinations are made almost daily, or in large numbers at some particular time, it is more convenient to remove the calcium hydroxide from a sufficient quantity of the cyanide solution to meet one's needs for a week. For each 100 cc. of solution, add up to 1 gm. of crystallized disodic phosphate, $Na_2PO_45H_2O$, in *finely powdered form*, shake, and filter or centrifuge.

This cyanide solution when used with the uric acid reagent described in the next section has never given us a single turbid result. But, at least at room temperatures, deterioration, in the sense of giving less color, is not completely inhibited. In a month, the chromophoric power of the cyanide will have diminished about as much as it was originally increased by the treatment with calcium oxide. At the lower temperatures of a refrigerator the deterioration is very much slower. As no one has ever before obtained anywhere near the maximum chromophoric power of the cyanide solutions, it obviously is not now essential to insist on having it. The cyanide solution described here will remain perfectly usable for at least 2 months, if kept at room temperatures, and for many months if kept in a refrigerator.

2. Uric Acid Reagent.—The preparation of the uric acid reagent completely free from phenol reagent was described quite recently (1), and it will therefore be described here only most briefly. Unless the uric acid reagent is free from phenol reagent it will not only give larger blanks with sheep blood filtrates from unlaked blood, but will also give some color at room temperatures, even with the most perfect cyanide solution.

Transfer 100 gm. of sodium tungstate and 200 cc. of water to a 500 cc. flask. Shake until the tungstate is dissolved. Add slowly, with shaking, and thorough cooling, 20 cc. of 85 per cent

phosphoric acid. Pass a rather slow H_2S current through the solution for 20 minutes, and during the process (at the end of 3 to 4 minutes) add another 10 cc. of 85 per cent phosphoric acid. Filter through Whatman No. 41 filter paper, and collect the first 40 cc. of filtrate separately, and pour them back on the filter.

Transfer the filtrate to a separatory funnel (capacity 1 liter) and shake for a couple of minutes with 300 cc. (1.5 volumes) of alcohol. The mixture separates into two layers. Transfer the lower layer immediately into a previously weighed 500 cc. flask. Discard the upper layer. Add water to the mixture in the weighed flask until the weights of the contents reach 300 gm.

Boil this solution a few minutes to remove the H_2S. Remove the flame, add 20 cc. of 85 per cent phosphoric acid and boil slowly but continuously for 1 hour, using a condenser to prevent concentration. At the end of an hour, remove the flame, and decolorize with a few drops of bromine. Boil off the excess bromine and cool.

Transfer 12 gm. of lithium carbonate to a 500 cc. beaker, add first 25 cc. of phosphoric acid, and then slowly 150 cc. of water. Boil to remove the CO_2 and see that the phosphoric acid actually has dissolved all of the carbonate. Cool the lithium phosphate solution, mix with the concentrated uric acid reagent, and dilute to 1 liter. Keep in well stoppered bottles protected from light.

This solution will retain its original slightly yellow color for months, if kept away from dust and light; but only a day or two of strong daylight will produce some blue color. The solution in daily use should therefore be kept in brown bottles.

3. Standard Uric Acid Solution.—About 5 years ago, I found that my standard uric acid solutions, which I believed to be thoroughly stable, had deteriorated. Some had kept only for a few months, others up to 2 years, but in time all became too weak. At that time, 5 years ago, I prepared another large series of uric acid solutions in order to find out the best proportions of formalin and acid which must be used. Some of these were soon discarded, and of the remainder, all but two were discarded last year. Of the remaining two, one is possibly 1 per cent too weak. It contains 25 cc. of formalin, and 3 cc. of glacial acetic acid per liter. The other one is still perfect, it contains 20 cc. of formalin, and 50 cc. of normal sulfuric acid per liter. I still have nearly 2 liters of each of these and shall continue to keep them.

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In the preparation of the standard uric acid solution described below, two slight changes are introduced, but the solution so prepared will certainly keep for at least 5 years. Weigh out on a watch-glass exactly 1 gm. of uric acid and transfer it to a not too small, dry funnel, inserted into a volumetric liter flask. Tap the funnel, so as to transfer nearly the whole of the uric acid to the flask. Transfer 0.6 gm. of lithium carbonate to a 250 cc. Florence flask, add 150 cc. of water; shake until solution is obtained (5 minutes). Some insoluble material remains and it is usually best to filter. Heat the solution or filtrate to 60°. Also, warm the liter flask under running warm water. Pour the warm lithium carbonate solution into the liter flask, incidentally washing into the flask the traces of uric acid which adhere to the watch-glass and funnel. Shake so as to dissolve the uric acid promptly, a little additional warming under hot tap water is permissible. The lithium carbonate solution is not always perfectly clear, even when filtered, and one should not mistake this little turbidity for undissolved uric acid and keep warming and shaking too long. In 5 minutes all of the uric acid should be dissolved. Shake the flask under running cold water without undue delay. Add 20 cc. of 40 per cent formalin, and half fill the flask with distilled water. Add a few drops of methyl orange solution and finally add, from a pipette, rather slowly and with shaking, 25 cc. of normal sulfuric acid. The solution should turn pink, while 2 or 3 cc. of acid are still left in the pipette, thus showing that the total acidity from adding 25 cc. of the acid is not great. Dilute to volume, mix thoroughly, and transfer to a clean, tightly stoppered bottle. This stock solution, containing 1 mg. of uric acid per cc. should be kept away from light.

To prepare the working standard, dilute 1 cc. of the stock solution, with water only, to 250 cc. It behaves exactly like a lithium carbonate solution of uric acid and in my laboratory this diluted solution keeps perfectly for many days.

Uric Acid Determination.

The details of the uric acid method described here were worked out with reference to blood filtrates prepared by the Folin-Wu method, but were found to work equally well with the new unlaked blood filtrates. There was a possibility that the increased sodium

sulfate content of the filtrates might prove disturbing either by yielding turbidities or by modifying the color reaction, but the sulfate is without effect. The method is, therefore, equally applicable to either filtrate.

Transfer 5 cc. of tungstic acid blood filtrate to a test-tube graduated at 25 cc., and add 3 cc. (+ 2 cc. of water) and 5 cc. respectively, of the standard uric acid solution to two other graduated test-tubes. To each tube add 5 cc. of the cyanide-urea solution and mix thoroughly. By means of a 5 cc. blood pipette, add 1 cc. of the uric acid reagent to each tube, mix well, and note the time. Let stand for 4 minutes, then heat in boiling water for 2 minutes, cool, dilute to volume, mix, and make the color comparison between the nearest standard and the unknown.

The 5 cc. standard corresponds to 4 mg. per cent of uric acid, and the 3 cc. standard to 2.4 mg. per cent.

It will be noted that in this determination the color development is allowed to proceed for 4 minutes before heat is applied. I have often tried to secure a longer waiting period than the 2 minutes which I formerly recommended, but invariably failed, because of the resulting turbidity. It was only by the combined action of urea and lithium salt that it became possible to lengthen the period. It is possible that others may consider the point involved of minor importance, but the fact is that the shorter the time between the starting of the reaction and the application of heat, the greater will be the blank, and the greater the blank, the narrower becomes the range of true proportionality for different amounts of uric acid. When 0.04 mg. of uric acid is now determined against 0.02 mg., and the latter is set at 20 mm. in the colorimeter, the 0.04 mg. will balance at 11 mm. instead of the theoretical 10 mm. This deviation from true proportionality is probably unavoidable with sodium cyanide as the only alkali.

A slightly better range of proportionality (20:10.6) can be obtained by the addition of some sodium hydroxide and using 2 cc. of the uric acid reagent, but under those conditions a new flaky form of sediment appears, and the process was abandoned. Without the extra alkali, 2 cc. of uric acid reagent cannot be used, because a much larger blank, due to liberated hydrocyanic acid, is obtained.

The analytical figures, recorded in Table I, are so clear that

TABLE I.

Comparison of Laked and Unlaked Blood with Respect to Non-Protein Nitrogen, Uric Acid, and Recovery of Added Uric Acid.

	Folin-Wu filtrate.			Unlaked blood extract.		
	Non-protein N.	Uric acid.	Recovery.	Non-protein N.	Uric acid.	Recovery.
	mg. per cent	*mg. per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*per cent*
Blood 1	30	2.5		21	2.3	
Same + 5 mg. per cent uric acid		6.4	78		7.5	104
Blood 2	32	2.7		21	2.5	
Same + 5 mg. per cent uric acid		6.3	71		7.5	100
Blood 3	43	2.5		28	2.2	
Same + 5 mg. per cent uric acid		6.2	75		7.2	100
Blood 4	30	2.2		18	2.0	
Same + 5 mg. per cent uric acid		5.9	74		7.0	100
Blood 5	30	3.0		17	2.7	
Same + 5 mg. per cent uric acid		6.5	70		7.8	102
Blood 6	22	1.3		15	0.8	
Same + 5 mg. per cent uric acid		4.7	68		5.8	100
Blood 7	35	2.9		20	2.5	
Same + 5 mg. per cent uric acid		6.1	64		7.4	98
Blood 8	36	1.5		20	1.5	
Same + 5 mg. per cent uric acid		4.9	68		6.4	98
Blood 9	29	2.0		19	2.0	
Same + 5 mg. per cent uric acid		5.6	72		6.8	96
Blood 10	30	2.3		20	1.7	
Same + 5 mg. per cent uric acid		5.9	72		6.7	100

very little in the way of interpretation needs to be added. The bloods were from patients of the Peter Bent Brigham Hospital. The small differences shown in the original uric acid content of the two sets of blood filtrates show how elusive even comparative analyses may be. It is only when the uric acid happens to come within that uninteresting average range of 2 to 3 mg. per cent, that the results are so nearly alike. Within this range, the large positive and negative errors happen to just about balance each other (when the uric acid reagent is free from phenol reagent). With abnormally high uric acid content, the Folin-Wu filtrates yield figures that are very much too low—as shown by the "recovery" figures. When the uric acid is very low, the reverse result is obtained. In one blood, the old form of filtrate gave 1.2 mg. per cent of uric acid, while the unlaked blood extract yielded only 0.5 mg. per cent. Whether or not much valuable information has been hidden by this deceptive character of the uric acid determinations, it is impossible to say, but it is certainly a fact that as yet there is not available any true picture of the fluctuations of uric acid in human blood.

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A COLORIMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF NITRATES AND NITRITES IN BIOLOGIC FLUIDS.

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An accurate method for the determination of nitrates in blood and urine is necessary for complete studies of nitrogen metabolism in patients to whom nitrates are given as diuretics.

Various colorimetric methods for the determination of nitrates have been described. Caron (1) used diphenylamine, but the results were widely variable. Letts and Rea (2) replaced diphenylamine with diphenylbenzidine. The resulting blue color, however, fades rather rapidly and colorimetric determinations could not be made. In the technique to be described in this paper, the uses of diphenylbenzidine and of sulfuric acid were retained. Otherwise, the method is substantially new. By this method it was possible to get a uniform development of color under conditions that permitted the colorimetric comparison of standard and unknown. The method permits the determination of 0.0003 mg. of nitrate nitrogen for each cc. of material and so is suitable for biologic fluids, such as blood, urine, edema and ascitic fluids. The essential elements in control of the development of color are prevention of rapid fading of color, control of temperature, and removal of protein.

Method.

Solutions and Reagents.—The following solutions are needed: (1) Mercuric chloride, 5 per cent. (2) Sodium carbonate, 1 per cent. (3) Standard potassium nitrate. To make this standard potassium nitrate solution, 0.3608 gm. of pure potassium nitrate is dissolved in distilled water, diluted to 1 liter, and thoroughly

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mixed. 1 cc. of this solution is diluted to 100 cc. 1 cc. of this diluted solution equals 0.0005 mg. of nitrate nitrogen. (4) Concentrated sulfuric acid, specific gravity 1.84. The acid must be free of nitrous and nitric acids. (5) Diphenylbenzidine. A good quality of this reagent can be purchased from the Eastman Kodak Company. 200 cc. of concentrated sulfuric acid are added to 50 mg. of diphenylbenzidine and 50 mg. of sodium chloride. A fresh supply of the reagent should be made up every 3 days, since such solutions slowly turn blue. After 3 days or so the reagent gradually loses its ability to produce the blue color in the colorimetric determination. The reagent is kept in the ice box.

Just before the reagent is used it is diluted with an equal quantity of distilled water. The dilution is carried on in an ice bath and care is taken not to add the reagent too rapidly. The diluted reagent should be cooled to room temperature before it is used.

Preparation of the Unknown Material.—The standard containing 0.0005 mg. of nitrate nitrogen for each cc. produces a depth of color which gives a satisfactory reading when set at 20 mm. in the colorimeter. It has been found more advisable to dilute the unknown to match this standard than to compare a definite dilution with a series of standards. Consequently several dilutions of the unknown are necessary.

If the unknown material is urine, and if toluene has been used as the preservative, it must be removed, for toluene prevents the development of color on the addition of diphenylbenzidine. 5 cc. of urine, made alkaline to litmus with sodium hydroxide, are evaporated to dryness on the steam bath. When the residue is dry it is transferred with as small a quantity of water as possible to a 100 cc. volumetric flask and acidified with 3 N HCl. Since a specimen of urine has occasionally been found which did not produce a blue color with diphenylbenzidine, even when considerable nitrate was known to be present, the procedure of removing the interfering substances with mercuric chloride has been adopted. 5 per cent mercuric chloride is added to the urine until a little, added to sodium carbonate in a spot plate, gives a yellow color. It is then made up to volume and filtered. If the solution is allowed to stand for some time (about 15 minutes) a clear filtrate is more readily obtained. From this filtrate a suitable dilution

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is made. Normal urine requires a dilution of from 1:10 to 1:25. If the patient has been given nitrates, much higher dilutions must be made.

It is better not to use toluene as a preservative and thus avoid the preliminary treatment necessary for its removal. Much better precipitation occurs if mercuric chloride is added to urine directly. The procedure for toluene-free urines is as follows: To 1 cc. of urine 5 cc. of $HgCl_2$ are added and the solution is then diluted and filtered. From the filtrate, higher dilutions are made.

If the unknown material is blood, mercuric chloride is used as the precipitant of protein. Considerable difficulty was experienced in trying to find a protein precipitant for plasma and whole blood. All the precipitants (tungstic acid, trichloroacetic acid, metaphosphoric acid, phosphotungstic acid, lead acetate, alcohol, colloidal iron, and aluminum cream) which were tried, with the exception of mercuric chloride, gave results which were too low.

To 1 cc. of plasma, serum, or ascitic fluid, in a 10 cc. volumetric flask, water is added to about 6 cc. Then 1 cc. of 5 per cent mercuric chloride is added. The mixture is made up to volume and shaken. If whole blood is to be used, it is necessary to add 1 cc. of 1 per cent sodium carbonate before the mixture is made up to volume. Suitable dilutions may be made from the filtrate.

Procedure.—For these determinations it is convenient to use Pyrex test-tubes, 180 by 20 mm., with ground glass stoppers. The test-tubes, each of which contains 1 cc. of the diluted filtrate of urine or blood, or 1 cc. of standard solution, are placed in an ice and salt freezing mixture. Then 10 cc. of the diluted diphenylbenzidine reagent are added by allowing it to run slowly down the side of the tube. The acid and nitrate solutions are mixed gently with a stirring rod. Then 10 cc. of concentrated sulfuric acid are added under the solution of nitrate and reagent. The tip of the pipette should be kept beneath the surface until all the acid has been added. The solutions are then mixed gently. It is important that the solutions be mixed with little or no rise in temperature. When the solutions have been thoroughly mixed and cooled, the tubes are transferred to a water bath that is at a temperature of about 50° and are allowed to remain there for 5 minutes, with occasional stirring, or until the solutions are

at about room temperature. They are then removed from the water bath and allowed to stand for 1 hour and 30 minutes. The results are read in the colorimeter (with the standard set at 20 mm.).

Calculation.—The following equation is employed.

$$\frac{20}{\text{Reading of unknown}} \times 0.0005 \times \text{dilution of unknown} \times 100 = \text{mg. nitrate N for each 100 cc. of material}$$

Precautions.—1. Because of the prevalence of nitrates in tap water, extra precautions should be taken in the cleaning of glassware. It is advisable to clean all glassware in cleaning acid, and to rinse thoroughly with distilled water.

2. Special care should be taken in mixing the reagent and nitrate solution, and in packing the tubes. It is convenient to use a freezing mixture of salt and ice. The tubes may be inserted into this with little danger of contamination and the outer surfaces of the tubes are evenly exposed to the cooling medium. If nitrites are present, even more care in mixing is necessary, for nitrites are distinctly unstable. The presence of nitrites can be detected by the immediate appearance of a blue color on the addition of the reagent.

3. A colorimeter with clear glass cups, the bottoms of which are fused on, should be used. This is advisable since such a concentrated solution of sulfuric acid is used.

DISCUSSION.

Fig. 1 shows the rate at which the color developed in the solution. There was a fairly uniform development which reached its maximum in about 1 hour and 20 minutes. The readings were taken with a spectrophotometer at intervals of a minute up to 83 minutes after the addition of the reagent. Readings taken at 100 and 130 minutes were the same as those taken at 83 minutes. Letting the solution stand for 90 minutes insured complete development of color, and the fact that the color did not begin to fade for at least 40 minutes after that gave ample time for reading.

When standards of different strengths were read against each other, the range of variation between the theoretic reading and the

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actual reading in thirteen determinations was from 0 to 1.2 mm. and the average 0.26 mm.

Table I shows the results of addition of potassium nitrate to urine, serum, plasma, whole blood, and ascitic fluid. The average error of fourteen determinations on urine, of which seven are given in Table I, was about 2 per cent. The average error of thirty-eight determinations, of which twenty-one are recorded in Table I, and which were performed variously on serum, plasma, whole blood, and ascitic fluid was less than 2 per cent. In the work with serum,

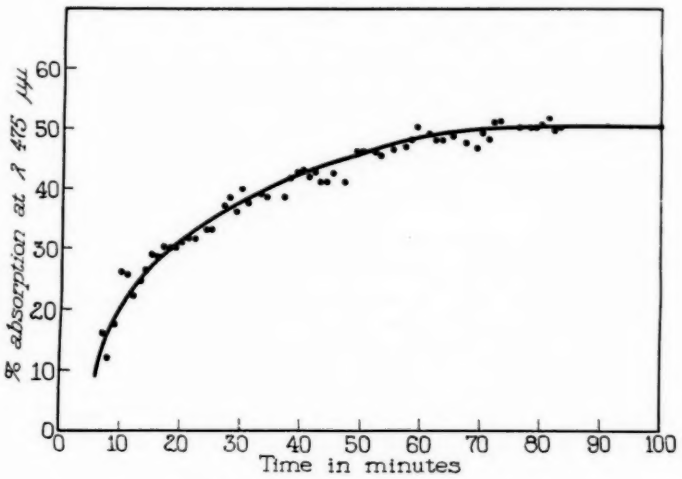

FIG. 1. Rate of development of color.

plasma, whole blood, and ascitic fluid, because of the preliminary addition of potassium nitrate, dilutions of 1:50 to 1:1000 were necessary.

Influence of Chloride.—Sodium chloride added to the diphenylbenzidine reagent intensified and stabilized the color developed, but the presence of an additional amount of chloride had no effect on the color. Addition of 1 cc. of 0.01 N sodium chloride, or 1 cc. of 0.1 N sodium chloride to standard solutions made no difference in color as read in the colorimeter.

Determination of Nitrite.—There has been some question as to the possibility of a portion of the ingested nitrate being excreted in

TABLE I.

Recovery of Nitrate Nitrogen Added to Blood, Ascitic Fluid, and Urine.

Sample No.	Nitrate N added per 100 cc.	Nitrate N recovered per 100 cc.	Difference.		Fluid to which nitrate was added.*
	mg.	*mg.*	*mg.*	*per cent*	
1	2.50	2.5			Serum.
	5.00	5.0			
	10.00	10.2	+0.2	+2.0	
	30.00	30.6	+0.6	+2.0	
	50.00	50.0			
2	2.50	2.42	−0.08	−3.2	Ascitic fluid.
	5.00	4.8	−0.20	−4.0	
	10.00	9.4	−0.60	−6.0	
	30.00	31.4	+1.40	+4.6	
	50.00	50.0			
3	5.00	5.0			Plasma (oxalated blood).
	10.00	10.0			
	30.00	29.3	−0.7	−2.3	
4	2.50	2.55	+0.05	+2.0	Oxalated whole blood.
	5.00	5.16	+0.16	+3.2	
	10.00	10.05	+0.05	+0.5	
	20.00	20.1	+0.10	+0.5	
	30.00	28.9	−1.10	−3.6	
5	5.00	4.93	−0.07	−1.4	Plasma.
	10.00	10.25	+0.25	+2.5	
	20.00	20.2	+0.2	+1.0	
6	50.00	50.0			Urine.
	100.00	100.0			
	50.0	48.0	−2.0	−4.0	
	50.0	49.0	−1.0	−2.0	
	100.0	96.0	−4.0	−4.0	
	100.0	96.0	−4.0	−4.0	
	50.0	52.0	+2.0	+4.0	

* The proteins were precipitated by mercuric chloride.

the urine as nitrite. It probably is not produced in any considerable amount.

The use of potassium permanganate for the purpose of oxidizing nitrite to nitrate and thus of determining the nitrite content of fluids by the difference between nitrate determined before and after oxidation has been suggested by Letts and Rea (2) and others. In my experience, the addition of potassium permanganate to urine results in the oxidation of other substances besides nitrites. Nitrogenous substances, such as ammonia, urea, and amino acids, perhaps are oxidized to nitrates by being heated with potassium permanganate in the presence of sulfuric acid. In favor of this hypothesis is the fact that normal solutions of ammonium chloride and of urea, which were negative for nitrates, gave a definitely positive reaction with diphenylbenzidine after being heated with potassium permanganate in acid solution.

Addition of the diphenylbenzidine reagent to a solution of pure silver nitrite gave the characteristic blue color. In order to determine whether the reaction was quantitative, the procedure for nitrates was followed through on a solution of silver nitrite containing 0.000455 mg. of nitrite nitrogen for each cc. Duplicate determinations gave the following results, 0.000453 and 0.00045 mg., with an accuracy of 99.3 per cent and 98.9 per cent, respectively. Titrations of this silver nitrite solution with 0.01 N potassium permanganate solution gave 49.7 and 50 mg. of silver nitrite. The theoretic value was 50 mg. of silver nitrite for each 100 cc. The method, therefore, is quantitative for nitrates and nitrites.

Mitchell, Shonle, and Grindley (3) reported some determinations of nitrates in the urine after the ingestion of potassium nitrate. The method they used was that of Schulze in which the nitrates are reduced to nitric oxide (NO) by ferrous chloride and hydrochloric acid; the nitric oxide evolved is collected over concentrated alkali and its volume measured. They claimed an accuracy of 95 to 100 per cent for pure nitrate solutions and of 93 to 94 per cent for urine. Since Schulze's method for nitrate determinations has been accepted as standard, it was thought advisable to check the diphenylbenzidine colorimetric determination against it. I tried to follow the technique of Mitchell, Shonle, and Grindley, but found that several modifications were necessary in the method of removing protein and extracting the

nitrates from urine before, in my hands at least, accurate results could be obtained.

A lead acetate filtrate, from which excess lead was removed with solid potassium carbonate, was used and found to yield theoretic results on potassium nitrate solutions, and on urine to which potassium nitrate had been added. Table II gives the results of comparison of the two methods.

Since good duplicate determinations on a solution at different dilutions constitute as excellent a check on a method as good recovery of added material, multiple determinations were made on 25 per cent potassium nitrate and sodium nitrate solutions and on urine, with at least two dilutions on each sample.

TABLE II.

Comparison of the Diphenylbenzidine Method with Schulze's Method.

Nitrate N, mg. per 100 cc. urine.		Difference.	
Diphenylbenzidine method.	Ferrous chloride and hydrochloric acid method (Schulze's).		
		mg.	*per cent*
34.5	33.5	+1.0	+3.0
23.3	24.6	−1.3	−5.4
27.0	27.4	−0.4	−1.5
21.3	20.4	+0.9	+4.3
25.6	24.4	+1.2	+4.8

In eleven cases, determinations were made in duplicate, in one case in triplicate, and in one case in quadruplicate. In each of these cases but one, the results of the readings with different dilutions corresponded to the third decimal place. In the one case in which correspondence was not so close, the quantities of nitrate and the consequent dilutions were nearly 40 times as large as in the general run of the determinations. Even in this case the error was less than 3 per cent.

CONCLUSIONS.

An accurate method for the determination of nitrates and nitrites, based on the development of a blue color by diphenylbenzidine, has been described. It has been applied to blood,

urine, ascitic fluid, pleural fluid, edema fluid, and saliva with an average error of ±2 per cent.

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1. Caron, M. H., *Ann. chim. anal.*, **17**, 9 (1912).
2. Letts, E. A., and Rea, F. N., *J. Chem. Soc.*, **105**, 1157 (1914).
3. Mitchell, H. H., Shonle, H. A., and Grindley, H. S., *J. Biol. Chem.*, **24**, 461 (1916).

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THE DIGITALIS GLUCOSIDES.

IV. THE CORRELATION OF GITOXIGENIN WITH DIGITOXIGENIN.

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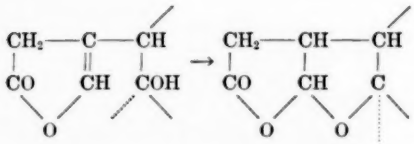
Digitoxigenin and gitoxigenin in glucosidic combination form the principal portion of the cardiac glucosides contained in the digitalis plant. Studies with both of these substances had recently reached the point where there was reason to assume a very close structural relationship between them. The analytical studies of Windaus and coworkers[1] and of the present writers[2] have shown digitoxigenin and gitoxigenin to be respectively $C_{23}H_{34}O_4$ and $C_{23}H_{34}O_5$. These substances contain only one double bond and are therefore derivatives of saturated tetracyclic structures. An additional close similarity between these substances has been found by the present writers in the demonstration that they are both $\Delta^{\beta,\gamma}$-lactones and, like the other aglucones of the strophanthidin series, may be isomerized to iso compounds.[2] The study of the mechanism of this rearrangement has shown that the portion of the molecule involved in the isomerization may be pictured as in the case of strophanthidin as follows:[3]

[1] Windaus, A., Westphal, K., and Stein, G., *Ber. chem. Ges.*, **61**, 1847 (1928). Windaus, A., and Stein, G., *Ber. chem. Ges.*, **61**, 2436 (1928).

[2] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **78**, 573 (1928); **79**, 553 (1928); **82**, 403 (1929).

[3] In view of observations which have been made since this manuscript was sent to press, the statement that gitoxigenin like digitoxigenin is isomerized to an iso compound must be qualified in the following manner. Results which will be published shortly now show that the oxidic ring of isogitoxigenin does not involve the tertiary hydroxyl group which is bridged in the case of isodigitoxigenin. Instead, the additional hydroxyl group is



In our previous communications certain pronounced abnormalities which were encountered in the study of isogitoxigenin and its derivatives, especially the unusual stability towards alkali of the lactone acid, isogitoxigenic acid, appeared to be explainable possibly on the assumption of the proximity to the lactone group of the extra hydroxyl of isogitoxigenin. After its removal as water, the resulting anhydroisogitoxigenic acid exhibited a normal saponifiability and resembled that of isodigitoxigenic acid. But the extent to which the structural resemblance of gitoxigenin to digitoxigenin would go could be only a matter of inference.

In order to determine this point, Windaus and coworkers have already pursued a definite program of work. This consisted in the formation by removal of the tertiary hydroxyl groups from each aglucone of a monoanhydro derivative from digitoxigenin and of a dianhydro derivative (digitaligenin) from gitoxigenin.

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involved by which gitoxigenin differs from digitoxigenin. Whereas digitoxigenin forms only a monobenzoate and gitoxigenin forms a dibenzoate, isogitoxigenin forms only a monobenzoate. Furthermore, whereas digitoxigenin, isodigitoxigenin, and isogitoxigenin form mono-ketones on oxidation with chromic acid of the identical secondary hydroxyl group in each to CO, in the case of gitoxigenin the extra hydroxyl group by which the latter differs from digitoxigenin is also oxidized to carbonyl so that this group cannot be tertiary as previously assumed. The so called gitoxigenon previously described (Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **79**, 557 (1928)), is a dicarbonyl compound, $C_{23}H_{30}O_5$. In addition the substance as previously obtained has been found to be a mixture of stereoisomers. Its behavior suggests the possibility that one of these CO groups may be aldehydic, in which case the extra hydroxyl group of gitoxigenin would, therefore, be of primary character.

The difference between the two oxidic rings of isodigitoxigenin and isogitoxigenin was not realized when the work here presented on correlation was begun. Although this difference may now affect the logic of a portion of the program presented in this paper, in particular that part in which the attempt was made to convert anhydroisogitoxigenin into isodigitoxigenin by hydrogenation, the final conclusions are correct as to the structural relationship between the two aglucones.

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The plan was then to hydrogenate the two double bonds in the former case and the three double bonds in the case of digitaligenin to form perhaps the identical saturated hydroxy lactone. Failing identity at this point, the remaining secondary hydroxyl group in each substance could be oxidized to carbonyl which could then in turn be replaced by CH_2 with the formation of the saturated lactones, $C_{23}H_{36}O_2$. In a series of articles[4] the results of these efforts have been recorded and although the desired chemical transformations were successfully accomplished the exact relationship of gitoxigenin to digitoxigenin was not disclosed by these experiments since the saturated lactones obtained from each substance proved to be not identical but isomeric. The possibility remained that the failure of the direct correlation of the two aglucones by this method was caused by the opportunity given for the formation of a number of isomers during the hydrogenation of so many double bonds, and that the substances which predominated in each case were different.

It appeared to us that the iso compounds might prove to be a more useful starting point for studies on correlation. Having once obtained evidence that isogitoxigenin is a normal iso compound,[3] the possibility appeared that its structural difference from isodigitoxigenin might consist solely in the possession of an extra hydroxyl group. In such a case the formation of an anhydroisogitoxigenin by removal of this hydroxyl group as water and subsequent hydrogenation should yield isodigitoxigenin. Unfortunately, only a portion of this program could be accomplished. When isogitoxigenin was dissolved in concentrated hydrochloric acid at low temperature (0°), it dissolved and was replaced by a crystalline chloro compound, *chloroisogitoxigenin*, in which the hydroxyl group in question[3] has been substituted by chlorine. When, however, the reaction was performed at 20°, although the chloro compound was formed as an intermediate stage, the final substance isolated was halogen-free, and on analysis proved to be *anhydroisogitoxigenin*. Unfortunately, all attempts to hydrogenate this substance after repeated recrystallization were unsuccessful, a result which is very difficult to understand in view of the work

[4] Windaus, A., and Bandte, G., *Ber. chem. Ges.*, **56**, 2001 (1923). Windaus, A., Bohne, A., and Schwieger, A., *Ber. chem. Ges.*, **57**, 1388 (1924). Windaus, A., and Freese, C., *Ber. chem. Ges.*, **58**, 2503 (1925).

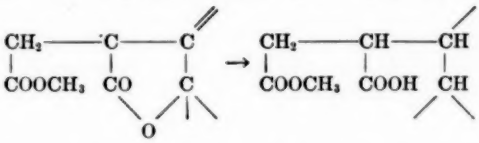
which will be described below. Apparently anhydroisogitoxigenin possesses a double bond which is very resistant to hydrogenation by the usual procedure. The formation of a new ring during the loss of water is greatly to be doubted. The attempt was also made to replace the chlorine in the chloro compound by hydrogen but with a similar lack of success. However, our main purpose was brought to a successful conclusion by a different procedure in which use was made of another isogitoxigenin derivative, isogitoxigenic acid.

When isogitoxigenic acid is treated with concentrated hydrochloric acid at ordinary temperature it dissolves with the formation of a crystalline *chloro-γ-isogitoxigenic acid* in which the tertiary hydroxyl group is replaced by the chlorine atom. Contrary to the experience with isogitoxigenin itself, the reaction appeared to stop at this point and no evidence of the subsequent formation of an anhydro acid was obtained. The latter was prepared by another step. The chlorine in the chloro acid proved to be labile towards alkali and depending upon the conditions employed could be replaced by hydroxyl or removed to form the unsaturated anhydro acid. Both of these reactions appeared to occur simultaneously but conditions were finally found in which either the one or the other reaction predominated. When the chloro acid was dissolved at 0° in a slight excess of very dilute ammonia, the chlorine was gradually replaced by hydroxyl. The resulting acid, however, in its optical behavior proved to be isomeric with the parent isogitoxigenic acid. Isomerization on a center of asymmetry has occurred under the influence of the strong acid which was used for the preparation of the chloro acid. This is apparently analogous to the observations which we have made in the case of the isomerization of α-isostrophanthic acid to γ-isostrophanthic acid,[5] and, as will be later shown, occurs also in the case of isodigitoxigenic acid. We have adopted, therefore, the designation γ to indicate the analogy of these isomerization products in the digitalis series with those obtained by similar means in the isostrophanthidin series.

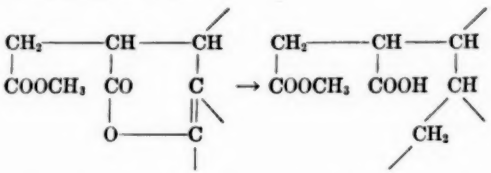
By the use of stronger alkali, hydrochloric acid is removed from the chloro acid with the formation of an unsaturated *anhydro-γ-*

[5] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 835 (1927).

isogitoxigenic acid.[6] It was hoped that this substance, contrary to anhydroisogitoxigenin, might yield to catalytic hydrogenation with the formation of *γ-isodigitoxigenic acid.*[3] As the methyl ester this anhydro derivative indeed absorbed hydrogen readily, but an unexpected complication was encountered in that the reaction did not stop at the normal 1 mol stage required by the one double bond assumed to be present. Instead 2 mols of hydrogen were absorbed and in accordance with this observation the resulting substance was found to possess the formula $C_{24}H_{38}O_5$. This substance on investigation proved to our great surprise to be the half ester (monomethyl ester) of a *dibasic acid,* $C_{22}H_{36}O_5$, which was readily obtained from it by saponification. Titration experiments showed that the lactone group of the parent anhydro acid had disappeared during the hydrogenation. In other words, while 1 mol of hydrogen was required to saturate the double bond the other was used for the cleavage of the lactone group by hydrogenation to the desoxy acid. This may be represented as follows:



At the present time we have under consideration as an alternative possibility the cleavage of an unsaturated δ-lactone which may be represented as follows:



The latter formulation has been suggested by certain observations which will be discussed in a later communication.[3] However, at the moment it may be stated that this type of cleavage appears to be attributable to the association of the double bond with the lactone ring since all attempts to cause a similar cleavage with the

[6] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **82**, 408 (1929).

saturated isogitoxigenic methyl ester were unsuccessful. A negative result was obtained also in the case of the saturated isodigitoxigenic methyl ester as well as its γ-isomer. A study of the factors involved in this type of cleavage is now in progress in this laboratory (W. A. Jacobs and A. B. Scott). During the present year, observations on a similar cleavage of lactone groups to the desoxy acid during catalytic hydrogenation have been reported by Mannich and Butz[7] in the case of certain unsaturated δ-lactones, by Borsche and Peitzsch[8] in the case of methysticin, and by LaForge and Smith[9] in the case of rotenone. On the other hand, although chloro-γ-isogitoxigenic acid is a saturated substance, when shaken with hydrogen and platinum oxide catalyst it yielded the above described half ester, so that the chlorine appeared to be replaced by hydrogen with simultaneous lactone cleavage. In this case, however, there is reason to believe that the chloro ester was first converted into the anhydro ester by removal of hydrochloric acid before hydrogenation occurred. The chloro ester has shown in general a distinct lability in this regard. On attempting to replace the halogen with hydrogen by the use of hydrochloric acid and amalgamated zinc, the ester of anhydro-γ-isogitoxigenic acid was obtained.

As previously discussed, the fact that the stability towards alkali of the lactone group of isogitoxigenic acid is greatly reduced on passing to the anhydro acid is probably referable to the proximity of the extra hydroxyl group. And now the observation that the unsaturated anhydroisogitoxigenic acid shows a behavior on catalytic hydrogenation which is not shown by the saturated substances is in agreement with the inferred proximity of the hydroxyl group and consequently of the double bond to the lactone group. It appeared for the moment that we were blocked in the attempt to correlate isogitoxigenin with isodigitoxigenin because of this abnormal course of the hydrogenation experiments. Fortunately, however, it has been possible to prepare the above dibasic acid also from isodigitoxigenin through the following series of substances.

Isodigitoxigenic acid on treatment with concentrated hydro-

[7] Mannich, C., and Butz, A., *Ber. chem. Ges.*, **62**, 461 (1929).
[8] Borsche, W., and Peitzsch, W., *Ber. chem. Ges.*, **62**, 360 (1929).
[9] LaForge, F. B., and Smith, L. E., *J. Am. Chem. Soc.*, **51**, 2574 (1929).

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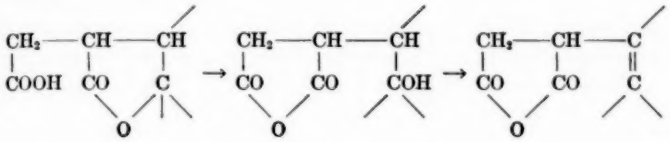
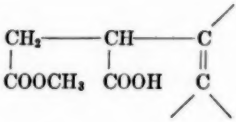
chloric acid was isomerized to *γ-isodigitoxigenic acid*. On heating this acid with a mixture of acetic anhydride and acetyl chloride the lactone group was opened with the formation of a substituted succinic anhydride and simultaneously the exposed tertiary hydroxyl group was removed as water. These transformations may be represented as follows:



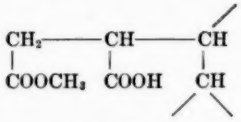
At the same time, the secondary hydroxyl group was acetylated. The formation of this substance, the *acetate of γ-digitoxenoldiacid anhydride*, paralleled exactly that of the unsaturated anhydride obtained from *β*-isostrophanthic lactone acid.[10] Contrary to our experience with the latter, however, the attempt to accomplish cleavage of the lactone group of the isodigitoxigenic acid by the use of methyl alcoholic hydrochloric acid instead of the above reagents was unsuccessful, due to the formation of obscure non-crystalline alteration products. Similarly, saponification experiments with alkali on the above unsaturated anhydride resulted in non-crystalline products which are probably referable to the instability of the unsaturated dibasic acid towards alkaline reagents. It was possible, however, by gentle treatment with methyl alcoholic hydrochloric acid to open the anhydride group with the formation of a half ester, the *acetate of γ-digitoxenoldiacid monomethyl ester*.



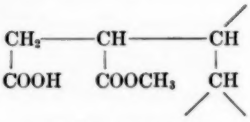
On hydrogenation this substance was readily converted into the *acetate of γ-digitoxanoldiacid monomethyl ester*. This saturated substance was now readily saponified by alkali with simultaneous removal of the acetyl group to the dibasic *γ-digitoxanoldiacid*. The

[10] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **84**, 183 (1929).

latter proved to be identical in all respects with the dibasic acid which resulted on saponification of the half ester obtained on hydrogenation of anhydro-γ-isogitoxigenic methyl ester. This was confirmed by the preparation of the identical *dimethyl ester* from both sources. Likewise, partial saponification of the dimethyl ester in each case gave rise to identical substances, the *stable monomethyl ester*. This stable half ester is isomeric with the half ester which had been obtained by hydrogenation of anhydroisogitoxigenic methyl ester. Since the latter is more readily saponified by alkali we have designated it as the *labile half ester*.

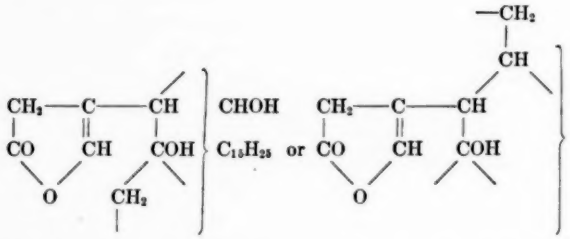


Labile half ester.

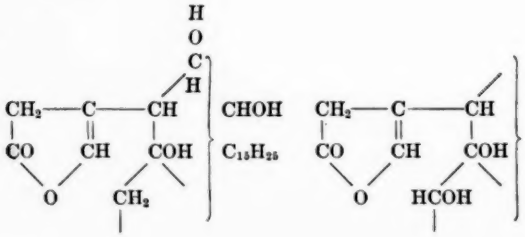


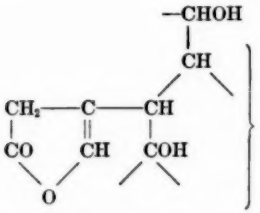
Stable half ester.

From these results it may be definitely concluded that gitoxigenin is hydroxydigitoxigenin. Digitoxigenin may be represented in partial formula as:



For gitoxigenin we have under consideration the following partial formulæ





in which the extra hydroxyl group of gitoxigenin may be secondary or primary. In the latter case the corresponding group of digitoxigenin would be methyl.

We are especially indebted to E. Merck, Darmstadt, and Merck and Company, Inc., of Rahway for their generous gift of the "digitoxin insoluble by-product" from which the material used in this research was prepared.

EXPERIMENTAL.

Derivatives of Isogitoxigenin.

Chloroisogitoxigenin.—0.4 gm. of isogitoxigenin was treated with 4 cc. of hydrochloric acid (1.19) at 0°. Solution rapidly occurred. On maintaining this temperature, a thick paste of crystals gradually formed. After 2 hours the mixture was diluted with ice water and the precipitate was collected with water. After washing free of acid, the dried substance was recrystallized by the addition of dry ether to the chloroform solution. It formed lustrous needles which melted at 167°. The melting point remained unchanged after repeated recrystallization from 95 per cent alcohol. Since the substance decomposed when maintained at an elevated temperature, the analyses were made on the desiccator-dried substance.

The results obtained were not satisfactory after repeated trials. This is probably attributable to the tenacious retention of solvent. However, since the exhaustive study of the substance would have caused a profitless waste of costly material, we have remained content with the data obtained.

4.633 mg. substance: 3.493 mg. H_2O, 11.300 mg. CO_2.
7.928 " " : 2.535 " AgCl.

$C_{23}H_{33}O_4Cl$.	Calculated.	C 67.53, H 8.14, Cl 8.68.
	Found.	" 66.51, " 8.43.
	"	Cl 7.90.

Anhydroisogitoxigenin.—0.5 gm. of isogitoxigenin was dissolved at 20° in 20 cc. of hydrochloric acid (1.19). Within a few minutes crystallization of the chloro compound began. This, however, on continued shaking redissolved and about 15 minutes later a new crystalline deposit began to separate. After several hours the mixture was carefully diluted and extracted with chloroform. The washed and dried extract on concentration gave a crystalline residue which was collected with 50 per cent alcohol.

When recrystallized from 95 per cent alcohol, it formed lustrous rectangular platelets which melted at 212°. It is soluble in alcohol, chloroform, and acetone, and but sparingly soluble in ether.

For analysis the substance was dried at 100° and 15 mm.

4.550 mg. substance: 3.534 mg. H_2O, 12.342 mg. CO_2.
$C_{23}H_{32}O_4$. Calculated. C 74.14, H 8.67.
Found. " 73.98, " 8.69.

Chloro-γ-Isogitoxigenic Acid.—2 gm. of isogitoxigenic acid were dissolved at 20° in 20 cc. of hydrochloric acid (1.19) as previously described.[6] The chloro acid which separated was collected after 2 hours with additional reagent and washed with water. The dried substance was recrystallized by solution in dry acetone and subsequent concentration to smaller volume. The acid crystallized as long, broad platelets which melted with decomposition at 255° after preliminary softening. It is appreciably soluble in methyl and ethyl alcohols and acetone, and but sparingly soluble in chloroform or ether. The alcoholic solution gradually deposits silver chloride when boiled with silver nitrate.

$$[\alpha]_D^{20} = -101° \text{ (c = 0.650 in 95 per cent alcohol).}$$

When dried at 75° under diminished pressure the substance did not lose appreciably in weight.

3.725 mg. substance: 2.530 mg. H_2O, 8.880 mg. CO_2.
9.275 " " : 3.010 " AgCl.
$C_{23}H_{33}O_5Cl$. Calculated. C 64.99, H 7.83, Cl 8.35.
Found. " 65.01, " 7.60.
" Cl 8.03.

Chloro-γ-Isogitoxigenic Methyl Ester.—A suspension of the acid in acetone was esterified with diazomethane. The ester formed

needles from dilute methyl alcohol which effervesced at 155° after preliminary softening and contained solvent. It is easily soluble in methyl and ethyl alcohols, acetone, and chloroform, and dissolves fairly readily in ether and benzene.

For analysis the substance was dried at 55–75° and 15 mm. When heated higher it gradually melted with decomposition.

4.577 mg. substance: 3.433 mg. H_2O, 11.030 mg. CO_2.
6.003 " " : 1.901 " AgCl.

$C_{24}H_{35}O_5Cl$.	Calculated.	C 65.64, H 8.04, Cl 8.08.
	Found.	" 65.71, " 8.38.
	"	Cl 7.84.

γ-Isogitoxigenic Acid.—0.7 gm. of chloroisogitoxigenic acid was suspended in 175 cc. of water and the mixture was treated with 0.5 cc. of concentrated ammonia. After being shaken to dissolve, the clear solution was allowed to stand for 2 days at 20°. Acidification with acetic acid caused the precipitation of a rapidly crystallizing acid which was collected with water and proved to be halogen-free. It was recrystallized by solution in a few cc. of 50 per cent alcohol with the aid of a drop of ammonia and subsequent acidification with acetic acid. The acid separated as broad, flat needles which were anhydrous and effervesced at 260°. It is soluble in descending order in methyl and ethyl alcohol and acetone, and very sparingly soluble in the other usual solvents.

$[\alpha]_D^{20} = -27°$ (c = 0.637 in 95 per cent alcohol).
3.378 mg. substance: 2.556 mg. H_2O, 8.455 mg. CO_2.

$C_{23}H_{34}O_6$.	Calculated.	C 67.99, H 8.44.
	Found.	" 68.25, " 8.46.

Anhydro-γ-Isogitoxigenic Acid.—1 gm. of chloroisogitoxigenic acid was treated with 25 cc. of a 5 per cent solution of sodium hydroxide in 50 per cent alcohol. A gelatinous mass of the salt formed. On being shaken at room temperature a clear solution gradually formed which tended to deposit a crystalline sodium salt. After 2 hours the mixture was carefully acidified with acetic acid and cautiously diluted. A copious crystallization of broad, flat prisms slowly deposited, which possessed the properties previously reported[6] except that now the substance after recrystallization was found to melt at 210° instead of 215°.

$[\alpha]_D^{20} = +61°$ (c = 0.673 in 95 per cent alcohol).

3.900 mg. substance: 2.965 mg. H_2O, 10.182 mg. CO_2.

$C_{23}H_{32}O_5$. Calculated. C 71.08, H 8.31.
Found. " 71.19, " 8.50.

Anhydro-γ-Isogitoxigenic Methyl Ester. The ester was obtained in the usual manner from the acid with diazomethane. Careful dilution of the methyl alcoholic solution caused the separation of stout platelets which melted at 151°. The substance is soluble in the usual solvents except ligroin.

For analysis the substance was dried at 100° and 15 mm.

4.583 mg. substance: 3.432 mg. H_2O, 12.005 mg. CO_2.

$C_{23}H_{34}O_5$. Calculated. C 71.59, H 8.52.
Found. " 71.44, " 8.38.

Hydrogenation of Anhydro-γ-Isogitoxigenic Methyl Ester. γ-Digitoxanoldiacid Monomethyl Ester (the Labile Half Ester).—0.7 gm. of anhydro-γ-isogitoxigenic methyl ester was hydrogenated in methyl alcoholic solution with 0.1 gm. of platinum oxide catalyst prepared according to Adams and Shriner. Following the prompt reduction of the catalyst, absorption completed itself within 5 minutes after approximately 2 mols of hydrogen had been absorbed. The filtrate from the catalyst yielded on concentration and careful dilution lustrous hexagonal leaflets which proved to be acid in character. After recrystallization by careful dilution of its solution in methyl alcohol the half ester melted constantly at 191–192° and contained solvent of crystallization. It is readily soluble in the alcohols and acetone and is appreciably soluble in chloroform, benzene, and ether. The substance dissolves readily in dilute ammonia or sodium carbonate. If, however, the latter reagent is too concentrated, the acid is replaced by the sparingly soluble sodium salt which redissolves only on great dilution.

For analysis the substance was dried at 100° and 15 mm.

4.753 mg. substance: 3.990 mg. H_2O, 12.335 mg. CO_2.
5.120 " " : 2.780 " AgI.

$C_{24}H_{38}O_5$. Calculated. C 70.88, H 9.43, OCH_3 7.63.
Found. " 70.78, " 9.39.
" OCH_3 7.17.

In experiments in which platinum black and colloidal palladium were used, absorption of 2 mols of hydrogen was also observed with the formation of the same half ester. In an experiment in which the absorption was interrupted at the 1 mol stage, half of the starting material was recovered and the other half was found to have been converted into the above half ester.

In an experiment on the catalytic hydrogenation of chloro-γ-isogitoxigenic methyl ester the reaction was found to take the same course, which involved cleavage of the chlorine as halogen acid with hydrogenation and cleavage of the lactone group. γ-Digitoxanoldiacid monomethyl ester was obtained in excellent yield. The product melted at 191° and agreed in all properties with the substance obtained from the anhydro ester.

3.955 mg. anhydrous substance: 3.415 mg. H_2O, 10.270 mg. CO_2.
Found. C 70.82, H 9.66.

The fact that the substance is a half ester was confirmed by titration.

For this purpose 14.800 mg. of substance were treated with 1 cc. of alcohol and titrated against phenolphthalein with 0.1 N NaOH. Found, 0.356 cc. Calculated for 1 equivalent, 0.364 cc.

2.6 cc. of 0.1 N NaOH were then added to the titration mixture. After being refluxed in an atmosphere of nitrogen for 3½ hours, the mixture was titrated back. Found, 0.354 cc. Calculated for 1 equivalent, 0.364 cc.

γ-Digitoxanoldiacid.—When the solution of the salt obtained by saponification of the above half ester was acidified with acetic acid, a jelly formed, which slowly crystallized as needles. When recrystallized from dilute methyl alcohol the acid formed lustrous leaflets which contained solvent. It melted with effervescence at 207°, although this point depended upon the rate of heating.

$[\alpha]_D^{20} = +5.0°$ (c = 0.700 in 95 per cent alcohol).

For analysis the substance was dried at 100° and 15 mm.

4.470 mg. substance: 3.660 mg. H_2O, 11.515 mg. CO_2.
$C_{23}H_{36}O_5$. Calculated. C 70.35, H 9.25.
Found. " 70.27, " 9.16.

γ-Digitoxanoldiacid Dimethyl Ester.—The half ester was completely esterified in acetone solution with diazomethane. From the concentrated methyl alcoholic solution it readily crystallized as lustrous, rectangular plates or leaflets which were anhydrous and melted at 156–157° after slight preliminary softening. The substance is readily soluble in the usual solvents.

$[\alpha]_D^{20} = +6.5°$ (c = 1.997 in chloroform).

4.319 mg. substance: 3.765 mg. H_2O, 11.330 mg. CO_2.

$C_{25}H_{40}O_5$. Calculated. C 71.38, H 9.59.
Found. " 71.54, " 9.75.

γ-Digitoxanoldiacid Monomethyl Ester (Stable Half Ester).—0.12 gm. of the dimethyl ester was suspended in 30 cc. of 50 per cent methyl alcohol and treated with 2 cc. of 10 per cent sodium hydroxide solution. On gentle warming, complete solution occurred within a fraction of a minute. On acidification with acetic acid, glistening leaflets of the stable half ester gradually separated. The substance was recrystallized by concentration of its solution in dry acetone and separated as pointed prisms and platelets. From methyl alcohol it forms characteristic, long, hexagonal platelets. It melts at 218° and is very triboelectric. It is appreciably soluble in methyl alcohol and acetic acid and but sparingly soluble in acetone, chloroform, and ether. The substance has properties which lend themselves well for the characterization of the parent acid. For analysis the substance was dried at 100° and 15 mm.

4.887 mg. substance: 4.090 mg. H_2O, 12.680 mg. CO_2.

$C_{24}H_{38}O_5$. Calculated. C 70.88, H 9.43.
Found. " 70.77, " 9.36.

Derivatives of Isodigitoxigenin.

Isodigitoxigenic Methyl Ester.—Isodigitoxigenic acid was esterified in acetone solution with diazomethane. After removal of the solvent, the residue was dissolved in a small volume of methyl alcohol. On the addition of a few drops of water the ester crystallized. After repetition of this recrystallization the substance formed flat needles or long, rectangular plates which melted at 174° and contained no solvent.

4.565 mg. substance: 3.715 mg. H_2O, 11.900 mg. CO_2.
$C_{24}H_{36}O_5$. Calculated. C 71.23, H 8.98.
Found. " 71.10, " 9.10.

All attempts at catalytic hydrogenation of carefully recrystallized samples of this ester resulted in recovery of unchanged material.

γ-Isodigitoxigenic Acid.—2.1 gm. of isodigitoxigenic acid were dissolved at 25° in 40 cc. of hydrochloric acid (1.19). The isomer started to crystallize within a few minutes. After standing for 1½ hours, the mixture was carefully diluted and the reaction product was collected with water. The acid was suspended in a small volume of 50 per cent alcohol and then brought into solution by the addition of a slight excess of ammonia. On careful reacidification with acetic acid the acid crystallized as hexagonal or pointed platelets. These were most conveniently collected with 33 per cent alcohol because of the great solubility of the substance in stronger alcohol. As so obtained, it contained 1 mol of water of crystallization and melted at 118° with decomposition.

$[\alpha]_D^{22} = +60°$ (c = 0.553 in 95 per cent alcohol).

For analysis the air-dry substance was dried at 100° and 15 mm.

4.609 mg. substance: 0.205 mg. H_2O.
$C_{23}H_{34}O_5 \cdot H_2O$. Calculated. H_2O 4.41. Found. H_2O 4.45.
3.735 mg. substance: 2.925 mg. H_2O, 9.615 mg. CO_2.
$C_{23}H_{34}O_5$. Calculated. C 70.72, H 8.78.
Found. " 70.21, " 8.77.

γ-Isodigitoxigenic Methyl Ester.—This was prepared in the usual manner with diazomethane. On careful dilution of its methyl alcoholic solution it formed lustrous leaflets which melted at 168° and contained no solvent.

3.683 mg. substance: 2.980 mg. H_2O, 9.632 mg. CO_2.
$C_{24}H_{36}O_5$. Calculated. C 71.23, H 8.98.
Found. " 71.32, " 9.05.

In attempts at the catalytic hydrogenation of this substance, it was recovered unchanged. When the attempt was made by heating the ester in 1 per cent methyl alcoholic hydrochloric acid to achieve lactone cleavage with the formation of an anhydro dimethyl ester only non-crystalline resin was recovered.

Acetate of γ-Digitoxenoldiacid Anhydride.—1 gm. of γ-isodigitoxigenic acid was heated in a sealed tube in a mixture of 18 cc. of acetic anhydride and 2 cc. of acetyl chloride for 15 hours. The clear solution was concentrated under diminished pressure to remove all reagent. This was aided by addition of small amounts of chloroform to the glassy residue and redistillation. The residue was finally dissolved in chloroform and the resulting solution was shaken out successively with dilute sodium carbonate solution and water. The dried chloroform solution of neutral material after concentration left a glassy residue which readily crystallized under methyl alcohol.

When recrystallized carefully from methyl alcohol it formed leaflets which melted at 182–184° after preliminary softening and contained no solvent.

3.970 mg. substance: 2.930 mg. H_2O, 10.530 mg. CO_2.
$C_{25}H_{34}O_5$. Calculated. C 72.42, H 8.27.
Found. " 72.34, " 8.26.

Attempts to obtain the free acid by saponification were unsuccessful apparently due to more deep seated chemical change under the influence of the alkali employed.

Acetate of γ-Digitoxenoldiacid Monomethyl Ester.—0.1 gm. of the above anhydride was treated with 2 cc. of a solution of 1 per cent hydrochloric acid in dry methyl alcohol and warmed very gently until all dissolved. This occurred fairly readily. The mixture was at once cooled and carefully diluted. On rubbing, the half ester rapidly crystallized and was collected with 70 per cent methyl alcohol. After recrystallization from dilute methyl alcohol, it formed leaflets which contained solvent and melted to a resin at 89°. It is very readily soluble in most solvents.

For analysis it was dried at 65° and finally at 75° and 15 mm.

5.115 mg. substance: 0.295 mg. H_2O.
$C_{26}H_{38}O_6 \cdot 1\frac{1}{2}\ H_2O$. Calculated. H_2O 5.71. Found. H_2O 5.77.
4.820 mg. substance: 3.700 mg. H_2O, 12.360 mg. CO_2.
5.385 " " : 2.960 " AgI.
$C_{26}H_{38}O_6$. Calculated. C 69.91, H 8.58, OCH_3 6.95.
Found. " 69.94, " 8.59.
" OCH_3 7.25.

Acetate of γ-Digitoxanoldiacid Monomethyl Ester.—The above

half ester was hydrogenated in methyl alcoholic solution with platinum oxide catalyst. Approximately 1 mol of hydrogen was absorbed after saturation of the catalyst. The saturated substance readily crystallized on concentration to a few cc. Recrystallized from a small volume of methyl alcohol, it formed four-sided leaflets which contained solvent and exhibited a very uncertain melting point. After repeated recrystallization it softened to a jelly at 80° which appeared to resolidify at about 130° and then melted again at 182°. Although the melting point was not satisfactory, the presence of isomers seemed to be excluded by the fact that recrystallization made no apparent change in the optical activity of the substance recovered.

$$[\alpha]_D^{20} = +4° \text{ (c = 0.67 in methyl alcohol).}$$

For analysis the substance was dried at 40° and 15 mm.

4.720 mg. substance: 3.905 mg. H_2O, 11.988 mg. CO_2.

$C_{26}H_{40}O_6$.	Calculated.	C 69.59, H 8.99.
	Found.	" 69.27, " 9.26.

γ-Digitoxanoldiacid.—0.2 gm. of the above saturated acetate was warmed for 1 hour in 10 cc. of methyl alcohol and 25 cc. of 0.4 per cent sodium hydroxide solution. Acidification with acetic acid caused the separation of lustrous needles. After recrystallization from dilute methyl alcohol, it formed leaflets which melted with effervescence at 207°. When mixed with the dibasic acid obtained from isogitoxigenin it showed no depression. In all other properties the two substances appeared to be identical.

For analysis the substance was dried at 100° and 15 mm.

4.190 mg. substance: 3.413 mg. H_2O, 10.783 mg. CO_2.

$C_{23}H_{36}O_5$.	Calculated.	C 70.35, H 9.25.
	Found.	" 70.19, " 9.12.

γ-Digitoxanoldiacid Dimethyl Ester.—The identity of the above diacid with that obtained from isogitoxigenin was further confirmed by the preparation of the dimethyl ester, which agreed in all properties with that previously described. It melted at 156–157° and showed no depression with the isogitoxigenin material.

$$[\alpha]_D^{20} = +7.5° \text{ (c = 2.003 in chloroform).}$$

4.067 mg. substance: 3.460 mg. H_2O, 10.640 mg. CO_2.

$C_{25}H_{40}O_5$.	Calculated.	C 71.38, H 9.59.
	Found.	" 71.35, " 9.52.

γ-Digitoxanoldiacid Monomethyl Ester (Stable Half Ester).—On partial saponification as previously described the dimethyl ester gave the stable half ester which also proved to be identical with the analogous isogitoxigenin derivative. The stable half ester formed long, hexagonal platelets from acetone which melted at 218°.

5.320 mg. substance: 4.462 mg. H_2O, 13.892 mg. CO_2.
4.476 " " : 2.503 " AgI.

$C_{24}H_{38}O_5$.	Calculated.	C 70.88, H 9.43,	OCH_3 7.63.
	Found.	" 71.21, " 9.38.	
	"		OCH_3 7.38.

A MODIFICATION OF SAKAGUCHI'S REACTION FOR THE QUANTITATIVE DETERMINATION OF ARGININE.

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(From the Department of Experimental Medicine, the University of Kansas, School of Medicine, Kansas City.)

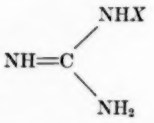
(Received for publication, December 27, 1929.)

The extremely sensitive color reaction given by certain guanidine derivatives with α-naphthol and sodium hypochlorite, which was observed by Sakaguchi (1925), has been extensively employed as a qualitative test for arginine. The greatest obstacle in a quantitative application of this reaction is its slow rate of color development with the probable destruction of some of the guanidine group by the hypochlorite. When sodium hypobromite is employed instead of sodium hypochlorite, the color development with arginine is practically instantaneous, although colorimetric readings are not possible, since the color begins to fade immediately and almost disappears in 10 minutes. However, the addition of urea immediately after the color development has taken place stabilizes the color sufficiently to permit colorimetric comparisons. This modification of Sakaguchi's reaction is being studied because of its possible application to the colorimetric determination of arginine and certain other guanidine derivatives.

While our studies are not as complete as those made by Poller (1926) on Sakaguchi's reaction, we found that arginine, glycocyamine, and methylguanidine give an intense red color with this modified reaction; while creatine, creatinine, urea, glycocyamidine, guanidine, and *as*-dimethylguanidine[1] do not give any color reaction. No compounds have been found that give the characteristic

[1] One commercial preparation of *as*-dimethylguanidine sulfate gave color corresponding to a probable content of methylguanidine sulfate as an impurity of 4.7 per cent. By recrystallization from 70 per cent alcohol, a preparation of *as*-dimethylguanidine sulfate was obtained which gave no color.

red coloration given by the reacting guanidine derivatives. It is perhaps safe to predict that derivatives having the following type formula



where X is either a fatty acid or alkyl radical, will produce color with this reagent.

Arginine shows a color reaction in a dilution of 1:2,500,000 or 0.0004 mg. per cc. Molar equivalents of arginine and methylguanidine produce equal amounts of color. Glycocyamine, or guanidine acetic acid, produces only 70 per cent as much color as would be expected when molar equivalents of methylguanidine and glycocyamine are compared. Since glycocyamine is an amphoteric compound, a part probably exists as an "inner salt" which does not respond to Sakaguchi's reaction.

Protein gives this color reaction because of its arginine content, but no colorimetric determination of arginine in unhydrolyzed protein is possible, since the color values are much less than the values expected from the actual arginine content of the protein. The application of the method to hydrolyzed protein is being studied. The fact that protein responds to this reaction makes it imperative that protein be absent from biological solutions being tested for arginine or other guanidine derivatives.

The modified reaction has been tested from two standpoints: first, the amounts of the various reagents and the conditions necessary to give maximum color production and the greatest accuracy in colorimetric readings; second, the effect of certain substances that are usually present or added during the preparation of solutions of biological material on the color development given by arginine.

The solutions used in this determination are as follows: (1) sodium hydroxide, 10 per cent; (2) α-naphthol, 0.02 per cent, made by diluting 20 cc. of 0.1 per cent α-naphthol in 95 per cent alcohol to 100 cc. with distilled water; (3) sodium hypobromite, made by dissolving 2 gm. of bromine in 100 cc. of 5 per cent sodium hydroxide; (4) urea, 40 per cent.

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The α-naphthol and hypobromite solutions are kept in amber bottles. The hypobromite solution will keep for about 2 weeks, while the α-naphthol solution will keep for at least 2 months.

The procedure is as follows: 5 cc. of the solution to be tested are placed in a test-tube kept in an ice bath. Add 1 cc. of sodium hydroxide and 1 cc. of α-naphthol and mix. The contents of the tube are cooled in the ice bath for 2 or 3 minutes, following which 0.1 to 0.2 cc. of sodium hypobromite is added, the tube shaken, and within 4 to 6 seconds, 1 cc. of urea is added and thoroughly mixed. The color is developed in the standard in the same manner and the colorimetric comparisons completed within 5 minutes.

Prior to performing the quantitative determination of arginine in an unknown solution, the amount of hypobromite necessary for complete color development must be determined. This is done by taking a series of tubes containing 5 cc. of the unknown and increasing the amount of hypobromite by 0.1 cc. portions. The standard should, however, contain only the amount of hypobromite necessary for complete color development, which is 0.1 cc. when a freshly prepared hypobromite solution is used.

The addition of various solutions is made more easily by using burettes. 10 cc. pipettes have been converted into pinch-cock burettes for this purpose.

The quantity of sodium hydroxide employed may be varied considerably if a sufficient amount is added to give the necessary alkalinity to the solution which is to be tested.

An excess of α-naphthol produces a yellow color, the intensity of which depends upon the amount used. However the quantity of α-naphthol necessary to give complete color development with the arginine concentrations employed, gives only the faintest trace of yellow and causes no disturbance in color matching, even when very dilute solutions are compared.

The greater the excess of hypobromite used in developing the color, the more quickly fading occurs, even if urea is added to destroy the excess hypobromite. When pure arginine solutions are tested, 0.1 cc. of hypobromite will give complete color development and, with the addition of an excess of urea, the color is stable for at least 10 minutes. With 0.2 cc. of hypobromite, readings must be made in 5 minutes to obtain comparable results. With

XUM

0.3 cc., the readings are 5 to 10 per cent too low and with 0.5 cc. hypobromite the color fading is too rapid for good comparisons when compared with a standard to which 0.1 cc. of hypobromite was added for color development.

The presence of substances like urea, creatinine, and reducing reagents which react with hypobromite, naturally requires the addition of more hypobromite for complete color development. However, the addition of an increased quantity of hypobromite in these cases does not appreciably influence the stability of the color produced as long as only the minimum quantity of hypobromite necessary for maximum color production is added.

TABLE I.

Determination of Arginine in Aqueous Solutions.

Arginine content of solutions.	Arginine found in individual determinations.				Maximum deviation from actual arginine content.	Deviation of average value from actual.
mg. per 1000 cc.	*mg. per 1000 cc.*	*mg. per 1000 cc.*	*mg. per 1000 cc.*	*mg. per 1000 cc.*	*per cent*	*per cent*
2.0	2.00	1.97	1.96	1.98	−2.00	−1.00
2.8	2.76	2.72	2.74		−2.86	−2.14
3.6	3.62	3.60	3.61		+0.55	+0.28
2.2	2.26	2.24	2.21	2.23	+2.73	+1.82
4.2	4.28	4.32	4.30	4.32	+2.86	+2.62
5.2	5.22	5.25	5.23	5.22	+0.96	+0.58
1.6	1.64	1.66	1.61	1.63	+3.75	+2.48
10.0	10.01	10.10	10.06	10.03	+1.00	+0.50

The addition of urea after the color development has taken place causes the color to become sufficiently stable to permit colorimetric readings. Without the addition of urea, the color produced with 0.02 mg. of arginine in 5 cc. and 0.3 cc. of hypobromite fades completely in 5 to 10 minutes, while with the addition of urea it shows only 5 to 10 per cent loss in color value. The greater the excess of urea added, the slower the rate of fading. For instance, the color produced when 0.5 cc. of hyprobromite is employed, fades in 2 to 3 minutes. If 0.5 cc. of 20 per cent urea is added, the color fades entirely in 20 to 30 minutes, but with 1 cc. of 40 per cent urea, the color does not entirely fade, even after 24 hours. The presence of urea does not completely prevent fading

but does slow the rate of fading sufficiently so that if colorimetric readings are made within 5 minutes, accurate comparisons can be made.

TABLE II.

Effect of Certain Compounds on the Color Production Given by Arginine.

Substance.	Amount added.	Arginine.	Color developed.
	mg. per 5 cc.	*mg. per 5 cc.*	*per cent*
Ammonium sulfate.*	0.1	0.01	45.5
	0.1	0.02	46.5
	0.05	0.02	71.5
	0.05	0.01	71.0
	0.02	0.02	91.0
	0.01	0.02	98.5
Urea.	4.0	0.02	52.6
	2.0	0.02	76.1
	2.0	0.04	76.1
	1.0	0.02	87.0
	0.5	0.02	100.0
Histidine dihydrochloride.	0.2	0.02	76.1
	0.2	0.04	78.1
	0.1	0.01	94.3
	0.1	0.02	95.2
	0.05	0.02	100.0
Creatine.	1.0	0.02	88.0
	0.5	0.01	100.0
	0.5	0.02	100.0
Creatinine.	2.0	0.01	100.0
	2.0	0.02	100.0
Uric acid.	0.2	0.02	100.0
	0.1	0.02	100.0
Glucose.	10.0	0.02	100.0
Glycerol.	20.0	0.02	100.0
	40.0	0.02	100.0
	60.0	0.02	90.0
Lysine monohydrochloride.	0.5	0.02	100.0
		0.04	100.0
Tryptophane.	0.1	0.02	100.0
Glycine.	1.0	0.02	100.0
Alanine.	1.0	0.02	100.0

* Ammonium sulfate values are given as the quantity of ammonium nitrogen present. Values for the other compounds are given as the weight of the compound actually employed.

It is necessary that the reaction be carried out at about 4°, because, at ordinary temperatures, the reactivity of the hypobro-

mite is so great that destruction of the guanidine group and of the color produced is very rapid, less color being obtained. The quantities of arginine that have been used vary between 0.005 mg. and 0.05 mg. per 5 cc. The volumes of the standard and the unknown must be the same since less color is developed with the same quantity of arginine, the greater the volume of solution. However, after the color has been developed it may be diluted with water.

The arginine content of solutions, which were made up by another member of the laboratory staff, was determined. Table I shows the results of individual determinations and shows the possibilities of this reaction as a colorimetric procedure for arginine.

Colorimetric determinations are influenced by the presence of other substances that occur in biological material. The reaction for arginine is profoundly influenced by the presence of certain substances, if they occur in sufficient concentration to render even a qualitative test of no value. Ammonia, histidine, tyrosine, and tryptophane produce a marked interference and, to a lesser degree, creatine and urea. Thus, the presence of either 0.3 mg. of ammonia nitrogen, 0.6 mg. of histidine dihydrochloride, 0.3 mg. of tyrosine, 0.3 mg. of tryptophane, 2.0 mg. of creatine, or 8.0 mg. of urea in 5 cc. with 0.01 to 0.04 mg. of arginine, either prevents all color formation or the color is so altered that even for qualitative purposes the test is worthless. With the exception of sulfides, sulfites, and iodides, no salts have been found which influence the color produced by arginine. Obviously metals forming insoluble hydroxides must be removed before the test can be performed.

Table II shows the amount of color developed by arginine in the presence of various compounds. Other compounds will probably be found that interfere, when the test is applied to various biological fluids. However, sufficient evidence has been presented to show the possibilities of this reaction as a method for the colorimetric determination of arginine.

CONCLUSION.

A modified Sakaguchi reaction is described which can be employed for the colorimetric determination of arginine.

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ENDOGENOUS URIC ACID AND HEMATOPOIESIS.

II. URIC ACID, RETICULOCYTES, AND ERYTHROCYTES AFTER HEMOLYSIS BY PHENYLHYDRAZINE HYDROCHLORIDE.

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The rôle of uric acid in the economy of the body has been the subject of many and varied investigations. The author has recently developed the hypothesis that this metabolite is produced from the nucleic acid resulting from the destruction of the nuclei extruded in the process of maturation of the erythrocytes. A series of determinations of the daily outputs of uric acid before and after severe hemorrhage showed a doubling for the period of regeneration followed by a gradual return to the basal level as the normal blood count was reached.

The present study was undertaken to test this hypothesis under the conditions of hemolysis and subsequent regeneration. Since the reticulated erythrocyte is considered to be the youngest red blood cell to be recognized in the peripheral circulation, a correlation of the uric acid outputs and the reticulocyte counts is to be expected. These counts were made daily, and several significant facts are established which tend to confirm the general hypothesis.

EXPERIMENTAL.

The daily uric acid outputs, erythrocyte counts, and reticulocyte counts were established for Dal Dale Doc, a thoroughbred Dalmatian coach dog, that was kept in a small experimental cage on a purine-free diet. After a control period of 21 days, the dog was given a capsule containing 50 mg. of phenylhydrazine HCl (Kahlbaum). During the next 10 days, this single dose reduced the red blood cell count from 5,709,000 to 3,320,000. Recovery

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was rapid; the normal blood level was reached in 9 days and surpassed on the 10th day by a count of 6,260,000. During the period of active regeneration, as indicated by a rise in the red blood

TABLE I.

Erythrocytes, Reticulocytes, and Uric Acid Output by a Dalmatian Coach Dog before and after Hemolysis by Phenylhydrazine HCl.

Date.	Red blood cell count.	Reticulocytes.		Uric acid.	Date.	Red blood cell count.	Reticulocytes.		Uric acid.
		per cent	*per c.mm.*	*mg.*			*per cent*	*per c.mm.*	*mg.*
Oct. 15	5,740,000	0.4	22,960	300	Nov. 5	4,840,000	0.5	24,200	300
" 16	5,500,000	0.4	22,000	256	" 6	4,580,000	0.8	36,600	527
" 17	5,670,000	0.3	17,010	313	" 7	4,330,000	1.4	60,620	300
" 18	5,390,000	0.2	10,780	274	" 8	4,650,000	1.5	69,750	624
" 19	5,920,000	0.2	11,840	227	" 9	4,930,000	2.2	108,460	266
" 20				260	" 10	4,570,000	1.5	68,550	400
" 21	5,820,000	0.4	23,280	240	" 11	4,050,000	0.9	36,450	640
" 22	5,740,000	0.6	34,440	202	" 12	3,430,000	3.0	102,900	545
" 23				184	" 13	3,600,000	1.4	50,400	288
" 24				328	" 14†	3,320,000	1.1	36,520	420
" 25		0.3		195	" 15	3,720,000	3.6	133,920	512
" 26		0.4		200	" 16	3,580,000	1.5	53,700	315
" 27				284	" 17	4,030,000	3.0	120,000	232
" 28		0.5		342	" 18	4,400,000	1.8	79,200	570
" 29		0.3		330	" 19	4,830,000	2.0	96,700	800
" 30		0.5		213	" 20	4,920,000	1.2	59,040	727
" 31		0.5		308	" 21	5,250,000	2.1	110,250	600
Nov. 1		0.4		216	" 22	5,400,000	3.6	194,400	408
" 2		0.5		200	" 23	5,770,000	0.7	40,300	328
" 3		0.4		192	" 24‡	6,260,000	1.0	62,600	380
" 4*	5,890,000	0.4	23,560	232	" 25	5,770,000	1.1	67,870	408
					" 26	5,880,000	0.8	47,040	571
					" 27	5,760,000	0.7	40,220	488
					" 28	5,460,000	1.1	60,660	408
					" 29	5,880,000	0.7	41,460	363
					" 30	5,720,000	0.3	17,160	326
					Dec. 1	5,660,000	0.8	45,280	320
					" 2	5,409,000	0.4	21,636	287
					" 3	5,490,000	0.7	38,430	355
Average...	5,709,000	0.4	22,836	265				66,377	436

* 50 mg. phenylhydrazine HCl by mouth.

† Maximum hemolysis.

‡ Maximum regeneration.

cell count, the uric acid increased from a basal level of 265 mg. to 481 mg., or 181 per cent.

A high uric acid level was also shown during the period of hemolysis. The marked marrow activity necessary to account for this output (432 mg.) is shown by the reticulocyte count which rose from a prehemolytic level of 22,836 per c.mm. to 69,990. Destruction and regeneration may thus go on side by side although one process may completely mask the end results of the other.

The data for the 21 days before and the 29 days after hemolysis are given in Table I.

TABLE II.

Erythrocyte Counts and Daily Uric Acid Outputs for a Dalmatian Coach Dog after Hemorrhage and after Hemolysis.

	Weight.	Lowest red blood cell count.	Highest red blood cell count.	Days to complete regeneration.	Average daily increase of red blood cell count.	Average daily uric acid.		Uric acid increase.	Total excess of uric acid above basal.	Basal level reached.
						Before treatment.	After treatment.			
	kg.	*million*	*million*			*mg.*	*mg.*	*per cent*	*mg.*	*days*
After 350 cc. hemorrhage	15	3.30	6.36	11	278,100	154	302	196	1738	21
After hemolysis by 50 mg. phenylhydrazine HCl	20.5	3.32	6.26	10	294,000	265	481	181	2160	18

DISCUSSION.

A. Comparison of Uric Acid Output after Hemorrhage and after Hemolysis.

In the previously published experiment, the red blood cell count after hemorrhage was 3,300,000, while after hemolysis the low level was 3,320,000. Regeneration was accomplished in approximately the same time in both instances, that is, in 11 days after hemorrhage and 10 days after hemolysis. The same high level was reached in each case; the count going to 6,360,000 after hemorrhage and 6,260,000 after hemolysis. The uric acid output above the basal level for the period of regeneration after hemorrhage was 1738 mg., and for the same period after hemolysis it was 2160

mg. The average daily output was increased 190 per cent after hemorrhage and 181 per cent after hemolysis. The daily uric acid output returned to the basal level 21 days after hemorrhage and 18 days after regeneration was started in hemolysis. The shorter interval in the second case may be due to the hyperplasia established during the period of regenerative activity masked by the red blood cell destruction.

The data for comparisons are given in Table II.

It will be noted that while the same dog was used in the two tests, the basal uric acid output was 154 mg. before hemorrhage and 265 mg. before hemolysis. In the interval between the two tests, *i.e.* from April, 1929 to October, 1929, the dog increased in weight from 15 to 20.5 kilos, the output being thus roughly proportional to the weight.

B. Action of Phenylhydrazine HCl.

A single dose of phenylhydrazine HCl administered orally continued to act as a hemolytic agent for a period of 10 days as shown by a falling red blood cell count. A similar action is reported by Jones (1911) for a period of 6 days following injection of phenylhydrazine in the rabbit. Whether the action was due to slow absorption or slow elimination was not determined. The blood was dark on the first 2 days, but did not show the brown color characteristic of phenylhydrazine injection. The dog seemed to suffer no embarrassment except on the 8th day which was characterized by loss of appetite and general depression. The urine was dark on the first 2 days but showed a normal color thereafter.

The sharp drop of the red blood cell count on the 1st day from 5,890,000 to 4,840,000 followed by a rapid decrease in destruction and a small compensatory increase, indicates a strong and immediate stimulus to the erythropoietic system, involving both the liberation factor (as shown by the reticulocyte count rise from 0.5 to 2.2 per cent) and the maturation factor (as shown by the rise in uric acid from 265 to 624 mg.).

That the subsequent drop to the low level was in part due to bone marrow injury is shown by the appearance of a few normoblasts in the peripheral circulation on the 3rd day. Their number increased and represented from 1 to 2 per cent of the total leucocyte count from the 7th to the 11th day. After this they gradually disappeared.

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C. Correlation of the Uric Acid Outputs and the Reticulocyte Counts.

It will be noted that both uric acid outputs and reticulocyte counts varied markedly for successive days after hemolysis. They present a series of peaks, which however are not simultaneous, but show an interval of lag varying from 3 to 4 days. The first peak, Peak A, for uric acid occurred on the 6th, while the first reticulocyte peak, Peak A′, came on the 9th day. Peaks B, B′; C, C′; D, D′; E, E′; and F, F′ follow in succession. The data are presented in Table III. The relative height of the peaks is also roughly proportional, as shown by the coefficient $\frac{R}{U}$ or

TABLE III.

Correlation of Uric Acid Output and Reticulocytes after Hemolysis by Phenylhydrazine HCl.

Uric acid.			Reticulocytes.			Days lag between peaks.	Coefficient $\frac{R}{U}$.*
Peak.	Date.	Output.	Peak.	Date.	Count.		
		mg.					
A	Nov. 6	527	A′	Nov. 9	108,460	3	205
B	" 8	624	B′	" 12	102,900	4	165
C	" 11	640	C′	" 15	133,920	4	204
D	" 15	512	D′	" 17–19	120,900	(3)	236
E	" 19	800	E′	" 22	194,000	3	243
F	" 26	570	F′	" 28	60,660	2	106

* The coefficient $\frac{R}{U}$ is the reticulocyte number divided by the mg. of uric acid.

reticulocyte number divided by mg. of uric acid. The values for Peaks F and F′ are relatively low and may represent a maturation of a reserve set of reticulocytes that are retained longer than those delivered to the peripheral circulation under the stress of a low red blood cell count.

D. Consideration of Reticulocyte Counts and Total Erythrocyte Regeneration.

If the total reticulocyte count in this series be compared to the total erythrocyte increase during the same period, it will be seen that the summation for the successive days from November 14 to

24 is far short of expectations. During this interval the total number of reticulocytes was 987,530 while the erythrocyte count increased by 2,940,000 giving an approximate ratio of 1:3. Examination of other series of reticulocytes and erythrocyte increases shows similar discrepancies. A consideration of the relative length of the reticulocyte period may account for these relationships.

On the assumption of a daily 1.5 per cent erythrocyte destruction, a like regeneration must also be postulated. Since the reticulocytes are identified as this young group of cells, they should be present in a concentration of 1.5 per cent. Since their normal value rarely exceeds 0.5 per cent, it follows that three generations must be born every 24 hours. The probability of the correctness of a short reticulocyte period is shown by the fluctuations occurring at 3 hour intervals in the work of Porter and Irving (1929) on liver therapy in pernicious anemia. On this hypothesis, the reticulocyte and erythrocyte values become consistent.

Calculations based on the reticulocyte percentages and the erythrocyte counts in the work of Minot, Murphy, and Stetson (1928) show reticulocyte outputs far in excess of the erythrocyte increase in given periods. In one case, the red blood cell count increased 200,000; the summation of the reticulocyte counts in the same time interval gives a value of 846,000. In a second case, the red blood cells increased 500,000 while the reticulocyte output was 1,424,000. These extremely high values may be interpreted as due to an unusually active marrow, the result of which is masked by a simultaneous hemolysis. The first 10 days after hemolysis in these experiments demonstrate such a relationship.

Another interpretation however may be put on the facts of high reticulocyte counts in cases of long standing. Eaton has shown that a marked hemorrhage is followed by a marked reticulocytosis. At a time interval corresponding to the length of life of the red blood cell, this crop of reticulocytes reaches senescence and is simultaneously destroyed, producing a "spontaneous anemia." This is followed by a second crop of reticulocytes, which in turn produces a second spontaneous anemia at their normal destruction. Thus a series of anemias is induced by a single hemorrhage. On this interpretation it follows that even a slight but continuous hemolysis will in time produce an extremely high proportional reticulocytosis, as 80 per cent reported by

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Sappington (1918), after the use of small daily doses of phenylhydrazine; or 56 per cent reported by Minot, Murphy, and Stetson (1928) in pernicious anemia.

E. Regeneration Rates after Hemorrhage and after Hemolysis.

It is generally stated that regeneration after hemolysis is more rapid than after hemorrhage because of the retention of hemoglobin which may be reutilized in the maturation of the erythrocyte. The results obtained in these two tests show little or no difference in regeneration rate. After hemorrhage the average daily increase was 278,100; after hemolysis the rate was 294,000. These rates may seem to be high, but are well within the range of similar values given by other investigators. Muir's data (1911) show increases of 420,000 after hemolytic serums in the rabbit; Jones (1911) reports rates of 460,000 after phenylhydrazine in the rabbit; Dawson's cases (1901) give 433,000 after hemorrhage in the dog; Starr (1928) reports increases of 170,000 per day after liver therapy in pernicious anemia.

F. Consideration of the Source of Endogenous Uric Acid.

The foregoing data on uric acid after hemolysis are highly confirmatory of the hypothesis that endogenous uric acid is produced in the bone marrow. This hypothesis has been independently developed and considered by Riddle (1929) following his work on uric acid output after the administration of liver extract in cases of pernicious anemia. Potent extracts increased the output; non-potent extracts had no effect.

Investigations by Emmel (1914) on the histology of the marrow of the pig indicate that the erythrocyte may become an enucleated cell by a process other than nuclear extrusion. In these investigations and in many others of a similar type, free nuclei enter the peripheral circulation and may be identified as such (Bunting (1906) and Jones (1911)).

That the marrow constitutes the principal organ of uric acid formation is shown indirectly by the work of Bollman, Mann, and Magath (1925), in their effort to show the seat of destruction of this metabolite in the common dog. They have shown a high and constant production of uric acid in dogs from which the entire livers have been removed, and show extremely high blood uric acid levels in dogs from which all abdominal viscera were taken.

Since the work of Zwarenstein (1928) on uric acid after exercise precludes the muscular system, and Peck and Thompson (1908) have shown very low outputs in neurasthenia, the marrow remains the only nuclear tissue sufficient in amount to account for the observed variations.

SUMMARY.

1. The average daily output of uric acid for a period of 21 days for a thoroughbred Dalmatian coach dog was 265 mg.; the reticulocyte count was 22,836 and the red blood cell count 5,709,000.

2. The average daily output of uric acid for 29 days after hemolysis with phenylhydrazine HCl was 436 mg.; the reticulocytes 66,377.

3. Regeneration rates and uric acid outputs after hemorrhage and after hemolysis are very similar.

4. Correlation of uric acid output and reticulocytes is shown and indicates a lag of from 3 to 4 days between maturation and extrusion.

5. The reticulocyte counts and total erythrocyte regeneration are considered on the hypothesis of an 8 hour span for the reticulocyte stage.

6. Additional confirmatory data are represented to support the hypothesis that endogenous uric acid arises from the extruded nuclei of the normoblast at the maturation of the erythrocyte.

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THE ISOELECTRIC POINT OF GELATIN AND ITS RELATION TO THE MINIMUM PHYSICAL PROPERTIES OF GELATIN.

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In a preliminary statement the writer (1) presented the proposition that the minimum physical properties of gelatin (conductivity, osmotic pressure, viscosity, maximum opacity) which are usually thought to define its isoelectric point are properties of pure gelatin, and that pure gelatin is not isoelectric; that pure gelatin from lime-conditioned stock is a weak acid which in an electrical field migrates to the anode; that the reactions necessary to bring pure gelatin to its isoelectric point are such as to indicate that isoelectric gelatin is an ionized salt of gelatin in which the positive and negative charges on the gelatin radical exactly balance each other (external rather than internal compensation of the charges on the gelatin molecule being assumed). In the present paper, experimental data and references are given in support of these assumptions.

Pure gelatin from lime-conditioned stock may be assumed to have the formula $X^{COOH}_{NH_2}$ (where X represents the main gelatin radical and the other groups are representative of the acid and basic groups) and should accordingly furnish the ions $(X^{COO^-}_{NH_3^+})^{H^+}_{OH^-}$. Since, however, this type of gelatin acts as a weak acid, the number of ionized groups furnishing H^+ ions would be greater and the ionized molecule may be represented by $(X^{COO^-}_{NH_2})^{H^+}$. The addition of a strong acid (HCl) to such gelatin will partly suppress its ionization as an acid and react with some of the NH_2 groups to form a hydrochloride of gelatin, $X^{COOH}_{NH_2Cl}$, which as a salt of gelatin would dissociate to form the more highly ionized

$(X^{COO^-}_{NH_3^+})^{H^+}_{Cl^-}$ just as the addition of HCl to a solution of ammonia forms the more highly ionized NH_4Cl. If the proper amount of acid is added to pure gelatin a point must be reached where the activity of $Cl^- + OH^-$ ions equals that of the H^+ ions. At that point the gelatin radical would have equal positive and negative charges and would be, according to the correct interpretation of the term, isoelectric. This point of view regarding the nature of the isoelectric point is the same as that of Michaelis (2) who states that "the logical general definition of the isoelectric point is given as that hydrion concentration at which an equal number of positive and negative ampholyte ions are present." When both positive and negative charges occur on the same molecule the isoelectric point is that point where the positive charges on the molecule equal the negative charges, as illustrated above. Reasons are given below why gelatin at its isoelectric point should be considered as an ionized salt of gelatin, and why its osmotic pressure, viscosity, etc. should be greater at the isoelectric point than in pure gelatin.

The assumption that the minimum physical properties of gelatin are not properties of the isoelectric point would at first hand appear contrary to the well established laws of ionic equilibria as applied by Donnan to membranes (3) and to proteins by Loeb (4). As will be shown in this paper, however, these same laws in conjunction with carefully obtained experimental facts can be applied to show the lack of any apparent relationship between the isoelectric point of gelatin and its minimum physical properties, and that these appear to belong to pure (*i.e.*, deashed) gelatin, which is not isoelectric.

So closely have minimum properties been associated with the isoelectric point that these have been made criteria of this state rather than any consideration of the state of electrical neutrality of the gelatin. Hitchcock (5), who has found that especially well purified gelatin has a minimum osmotic pressure and a maximum opacity at a pH of 5.05, has inferred that this must be the true isoelectric point rather than that previously established at pH 4.7. This inference would only be correct when based on the assumption that the state of aggregation of gelatin molecules in such pure gelatin is the same as it is in gelatin which is at its isoelectric point. Pure gelatin such as he prepared is, however, negatively

charged and, when under the influence of an electrical field, migrates to the anode. That there is a varied state of aggregation of gelatin at a varied pH, reaching a maximum state of aggregation at pH 4.95, the pH of pure gelatin, was shown by Kraemer and Dexter (6) who studied the light-scattering capacity of gelatin solutions.

The determination of the isoelectric point by cataphoresis is a usually accepted method. This method cannot, however, determine that the minimum physical properties are properties of the isoelectric point. That they should be such is arrived at by theoretical considerations. It should, therefore, readily be shown by other methods than by cataphoresis that pure gelatin is not isoelectric.

Loeb has shown that the effect of neutral salts on the properties of gelatin can be derived from the Donnan theory of membrane equilibria and that this effect must of necessity be at a minimum at the isoelectric point. One of the simplest and most accurate methods of demonstrating this effect is that of viscosity determinations.

Loeb, in his classic studies, found only a depressing action of neutral salts on the physical properties of gelatin, and concluded that, at any point of hydrogen ion concentration other than that of the isoelectric point, this cannot be otherwise, and found this to be a natural consequence of the theory of membrane equilibria. His studies with neutral salts were limited to points of hydrogen ion concentration which are comparatively distant from the pH of the isoelectric point, and from that of pure gelatin. His conclusions would, moreover, only be correct if it were true that isoelectric gelatin is the same as pure gelatin.

The present report presents experimental data to support the conclusion that aside from the demands of any theoretical considerations, pure gelatin is not at its isoelectric point and that gelatin would appear to have at least two isoelectric points.

It has been found in the present investigation that sodium chloride, added to gelatin solutions, accentuates rather than depresses the viscosity as long as the pH of these gelatin solutions is between 5.26 and 4.68. At these points the effect of the salt is nil and beyond them it has the depressing action which Loeb has found.

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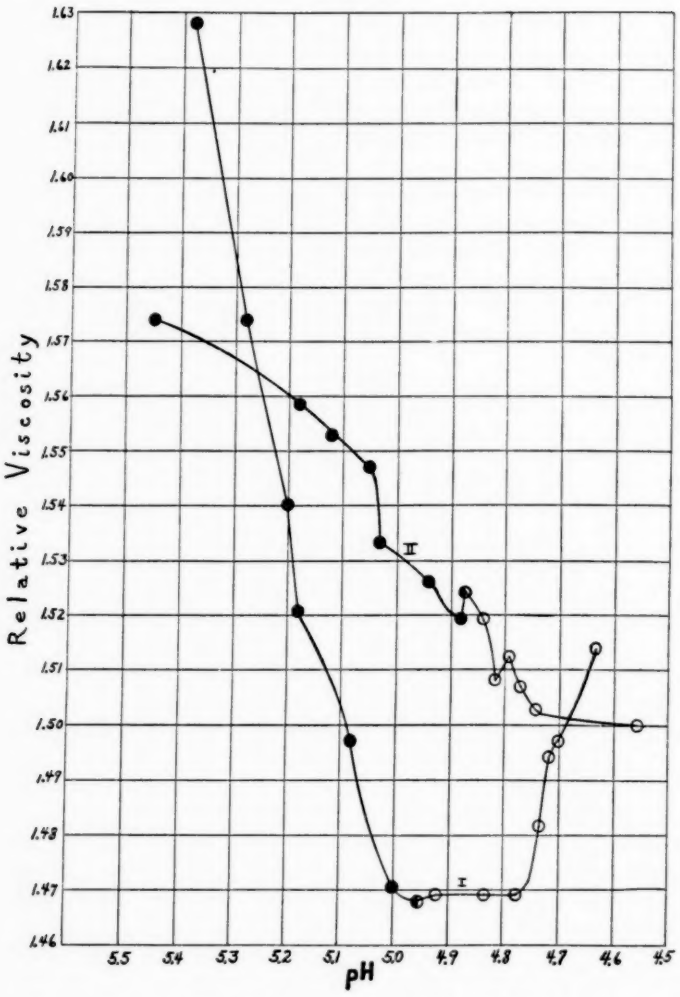

FIG. 1. Curve I, viscosities of 1 per cent solutions of gelatin after the addition of variable amounts of acid or base. Curve II, similar data obtained in the presence of $\frac{1}{32}$ N NaCl. A depressing effect of NaCl upon the viscosity is to be noted between pH 4.68 and 5.26, the points where the two curves cross. The solid dot indicates the viscosities after NaOH is added; the clear circle, viscosities after HCl is added.

These facts are illustrated by the data represented by Fig. 1. These data were obtained with 1 per cent solutions of pure gelatin having an initial pH of 4.95. Curve I represents the relative viscosity of these solutions after varying the pH by the addition of either HCl or NaOH. Curve II represents similar data obtained after adding to such solutions sufficient sodium chloride to made them $\frac{1}{32}$ N (the limiting concentration advised by Loeb in his experiments) with respect to this salt. The ordinates are the relative viscosities and the abscissæ the relative pH of these solutions.

It is to be noted that upon the addition of increasing amounts of HCl to pure gelatin of pH 4.95, the viscosity remains constant until a pH of somewhat less than 4.8 has been reached and then increases rapidly when still more acid is added. The addition of NaOH to pure gelatin causes an almost immediate rapid increase in viscosity.

The data of Curve II show that within the limits of a pH of 4.68 and one of 5.26, the presence of NaCl causes an accentuation of the viscosity. If the curves showing the influence of pH on pure gelatin and on gelatin plus NaCl cross at these points, then these points should represent the conditions of hydrogen ion concentration where the properties of solutions of pure gelatin and of solutions of gelatin and salt are identical. At pH 4.68 and 5.26, the effect of NaCl upon the viscosity is nil, and beyond these points NaCl depresses the viscosity. These points (4.68 and 5.26) should then, according to theory, represent the isoelectric points of gelatin. The nature of Curves I and II defines these two points rather sharply. The accuracy of these experiments, as indicated below, would place these isoelectric points within 0.01 or 0.02 of a pH of those which have been given.

The titration curves of Fig. 2 show the manner in which small increments of acid or base, when added to 1 per cent solutions of gelatin, affect the pH of these solutions, both in the absence and in the presence of sodium chloride ($\frac{1}{32}$ N) within the limits of the two isoelectric points. The successive increments were 0.1 cc. of 0.1 N acid or base per 100 cc. of gelatin solutions. It may be noted that the crossing points of the acid titration curves practically coincide with the sharp breaks in Curve II of Fig. 1, and that when solutions of pure gelatin are progressively made

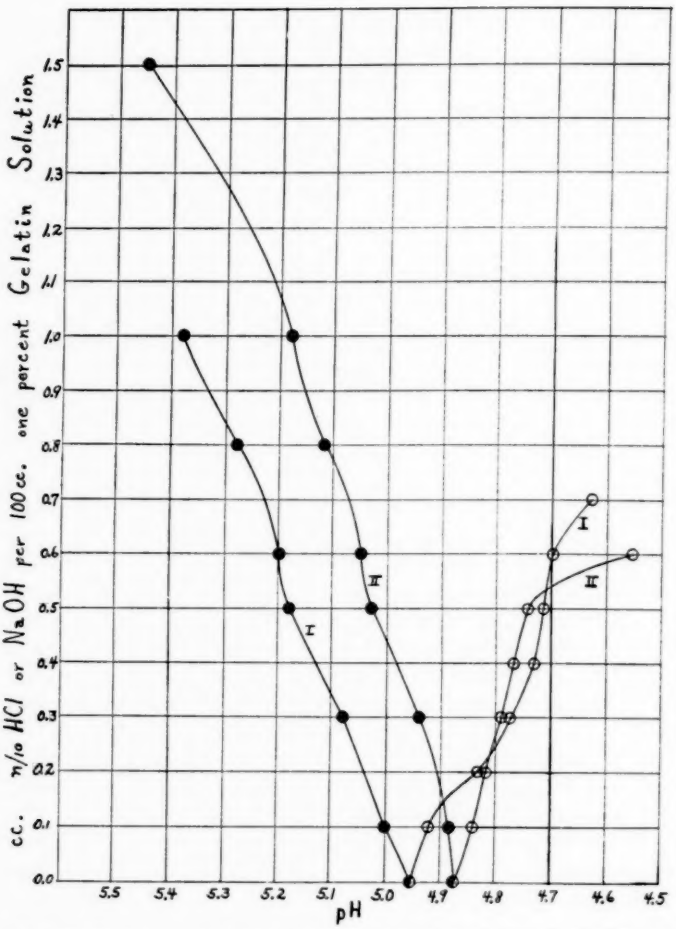

FIG. 2. Titration curves of 1 per cent gelatin solutions with NaOH and HCl. Curve I indicates change of pH by addition of acid or base; Curve II, similar change in the presence of $\frac{1}{32}$ N NaCl. The solid dot indicates the pH after the addition of NaOH; the clear circle indicates the pH after the addition of HCl.

more alkaline the presence of NaCl consistently decreases the pH, and when solutions of pure gelatin are made more acid the

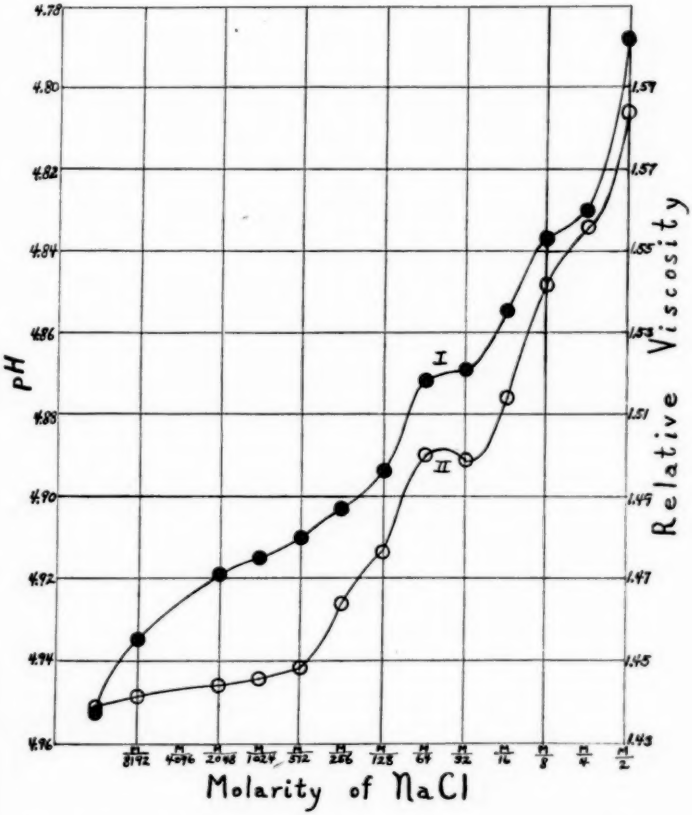

FIG. 3. Curve I shows the change in pH of 1 per cent solutions of pure gelatin due to the presence of variable amounts of NaCl; Curve II, the viscosities of these solutions.

presence of salt at first decreases the pH, then increases it, and finally decreases it again. The NaOH titration curves would indicate the lack of a pronounced variation in the dissociation

constants of the acid groups of gelatin. The HCl titration curves would, however, indicate the presence of an outstanding[1] basic group in gelatin.

The manner in which amounts of NaCl varying from 0 to 0.5 N affect the pH and viscosity of 1 per cent solutions of pure gelatin is shown by Fig. 3. The many inflections in the viscosity curve are consistently reflected by the subsequently determined hydrogen ion concentrations given in Curve II. It is remarkable that the change from $\frac{1}{64}$ to $\frac{1}{32}$ N causes no marked change in either viscosity or pH. All of the data of any one figure were obtained with the same solution of gelatin.

Experimental Methods.

The gelatin used in these experiments was almost completely deashed by the method of Northrop and Kunitz (7) and then further treated by the method heretofore used by the writer (8). By the former method almost all ionic impurities are removed by the dialysis of powdered gelatin under controlled conditions of hydrogen ion concentration, and by the latter small additional amounts are removed by electrolysis. For this purpose a 15 to 20 per cent gelatin gel is allowed to set in the lower half of an open Pyrex glass tube. The other half of the tube is filled with distilled water and the tube placed in a Pyrex beaker filled with distilled water. Platinum electrodes are placed about 1 cm. distant from the ends of the gel and a current of 400 volts passed through. During the electrolysis the temperature of the gelatin is kept at nearly 0°. The water is frequently changed and the electrolysis continued for about 36 hours. A section of gelatin about 1 cm. deep, nearest the electrodes, was discarded. (Essex XXXX powdered gelatin was used in these experiments.)

This latter process was invariably found to remove additional electrolytes from the gelatin and to decrease its conductivity. 1 gm. of such gelatin leaves no weighable ash when ignited. A 1.7 per cent solution was found to have a specific conductivity

[1] On the assumption of one of these groups for each molecule of gelatin, the sharp inflection of this curve would almost justify the calculation of the molecular weight of gelatin from these titration data. The requirement of 0.2 cc. of 0.1 N HCl to change 100 cc. of 1 per cent gelatin from pH 4.95 to 4.81 would indicate a molecular weight of gelatin of approximately 50,000.

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of 1.50×10^{-5}. 1 per cent solutions were repeatedly found to have a pH of 4.95. The water used in making up solutions of gelatin had a specific conductivity varying from 0.8 to 1.2×10^{-6}.

pH Determination.—The pH determinations[2] were made with the hydrogen electrode, with a Clark-Cullen rocking electrode (9), the refinement and precaution described elsewhere (10) being observed. The determinations were made at 37° ± 0.03°, in an air thermostat, with the actual temperature in the cells controlled by the small thermometer used with this type of cell. The liquid junction was saturated KCl with an open stop-cock, without the use of agar, since experience in this laboratory indicates that more consistent results are obtained without the use of agar. Most of the determinations were made in quadruplicate, the remainder in triplicate. These usually checked within 0.001 pH and the results reported are accurate to about 0.005 pH. Although for this work the absolute pH values are not as important as the relative value, for purpose of comparison it should be stated what the standard of reference was, since most of the present data concerning "isoelectric" phenomena of gelatin are confusing because no mention is made of the pH standardization used. In this laboratory all pH determinations are based on specially prepared 0.1 N HCl with an assigned value of pH 1.08 (11). The platinum electrodes were cleaned with hot water and were standardized, before and after each run, with standard phosphate solutions previously standardized against the 0.1 N HCl. The pH values are reported as pH values rather than as αpH for the reasons summarized by Robinson (12).

The viscosity determinations were made at 37° ±0.03°. These were made with an Ostwald viscosimeter after the solutions had remained in the thermostat for ½ hour. Aside from temperature equilibrium, it was found essential to allow the gelatin solutions to remain in contact with the capillary of the viscosimeter for some time before taking readings. At no time in the purification of the gelatin or in the preparation of the solutions was this material heated above 40°. This precaution was taken because it was previously found, while making conductivity determinations, that even at 50° the conductivity gradually increases, indicating

[2] The writer gratefully acknowledges his indebtedness to his colleague, Dr. I. P. Earle, for carrying out the pH determinations.

a possible hydrolytic change in the gelatin, when its solutions are subjected to this temperature. The time of outflow of solutions was measured by a stop-watch and the average of five readings taken. These seldom varied more than $\frac{1}{5}$ second. Solutions for which a series of readings varied more than $\frac{2}{5}$ second were refiltered and after thoroughly recleaning the viscosimeter, the viscosity was determined again. The relative value of the viscosity determination is based on the viscosity of water at 37° as unity.

DISCUSSION AND CONCLUSIONS.

On the assumption that Loeb's derivations of the physical properties of proteins at their isoelectric point as a natural consequence of Donnan's law of ionic equilibria are correct, it would follow that in order to satisfy this law, gelatin must exist in the form of associated molecules within the limits of pH bounded by its apparent isoelectric points. The fact that its opacity becomes greatest when its state of purity is greatest would make it seem that this opacity is due to the association of gelatin molecules. This point of view finds support in the previously quoted results of Kraemer and Dexter who find a maximum scattering of light by 1 per cent solutions of highly deashed gelatin at a pH of 4.95, indicating a maximum state of aggregation at this point.

That gelatin should become more associated as it is deprived of the last traces of its electrolytes is not difficult to conceive. The gelatin molecule contains both positive and negative charges and these are present in unequal numbers at any pH except at the isoelectric point. It is generally assumed that where either of these charges is in excess of the opposite kind, this excess is compensated by foreign ions of an opposite charge existing in the same solution but that, to the extent to which it is possible, positive charges are compensated by negative charges existing on the same molecule. Although internal compensation may occur, it would (in view of the possible remoteness of opposite charges on the same molecule) appear just as possible that all charges on any molecule could be compensated by the opposite charges of other ionized molecules in the same solution when such are present. Neutral salts apparently can form an electrical double layer around the gelatin molecule by the attraction of both ions of the salt molecule by the two opposite charges on the gelatin molecule.

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This point of view is not so different from that held by Pauli (13). When other ions are not present it would be just as probable that the charges on gelatin are compensated by intermolecular attraction as by the internal compensation of charges on the same molecule, a procedure which would tend to bring about a condition of aggregation of molecules. The addition of a salt to pure gelatin would, aside from affecting the activity of gelatin, provide for compensation other than that between gelatin molecules, and cause gelatin to exist in a more highly dissociated state. Even at the isoelectric point external rather than internal compensation would seem the more logical, and gelatin at its isoelectric point would therefore exist as an ionized salt which is compensated by equal numbers of positive and negative ions of foreign electrolytes and thus exist in a more highly ionized state than the associated molecules of pure gelatin. The greater solubility of some proteins, such as the globulins, in the presence of salts is evidence of such a compensatory mechanism.

There is no reason to be surprised that different samples of gelatin have a different isoelectric point since it is possible that a given gelatin is not a pure substance. It has been shown, Kunitz and Northrop (14), that such may indeed be the case. However, it is difficult to explain how a given sample of gelatin should have two isoelectric points, both below pH 7, and on opposite sides of the pH of maximum purity. Although the explanations for the behavior of gelatin just given might readily be adapted to an isoelectric point at pH 4.68, they would not apply to that found at pH 5.26. It was shown above how the addition of the proper amount of acid to the type of gelatin used in these experiments must bring it to its isoelectric point. No similar reasoning can be applied to show why the addition of a base to the same type of gelatin should bring it to a second isoelectric point. There is the possibility that the deductions which lead to the conclusions that the action of neutral salts on the properties of proteins must determine their isoelectric point are no longer tenable, because the variable state of aggregation of proteins under different conditions of hydrogen ion concentration has not been taken into account in arriving at the conclusions reached regarding this phenomenon.

It remains to be seen whether varying the concentration or the

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nature of the salt used in thus determining the isoelectric points of gelatin will result in always finding these at the same points. If this should not be the case then it would seem that the isoelectric point is of an indeterminate nature and that the present methods of its determination could only give results relative to the conditions of the determination. Although it may be possible to determine the isoelectric point in a buffered solution it would not seem possible to determine the minimum physical properties of gelatin under such conditions.

The determination of the osmotic pressure of gelatin is so important that the following precaution against possible error should be noted. In determining the osmotic pressure of gelatin solutions with the aid of capillary tubes in which water will rise to a height of several mm., a correction should be made for the comparatively low surface tension of 1 per cent gelatin solutions, as otherwise a relatively large error may result, especially at that point where the osmotic pressure is at a minimum.

SUMMARY.

The action of salts on the properties of gelatin has been applied for the determination of the isoelectric points of gelatin. It has been shown by the action of neutral salts on the viscosity of gelatin solutions that gelatin apparently has two isoelectric points, one at a pH of 4.68 and a second at a pH of 5.26.

It has been shown that all of the minimum physical properties of gelatin are therefore properties of pure gelatin, and not of isoelectric gelatin and that pure gelatin has a pH of approximately 4.95. Any inconsistency between these facts and the requirements of the law of ionic equilibria is explained on the basis of the existence of associated gelatin molecules between a pH of 4.68 and 5.26, the apparent isoelectric points of gelatin. No immediate explanation can be given for what appears to be an isoelectric point at pH 5.26. If this is not an actual isoelectric point then the derivation of the isoelectric point from the effect of neutral salts on proteins is held to be no longer tenable.

Aside from any effect which neutral salts may have on the activity of free acid or basic groups, the action of neutral salts on gelatin is explained as being that of furnishing ions for compensating the charges which ionized gelatin bears, and which in

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the absence of these salts tend to require intermolecular compensation, an explanation which is confirmed by the precipitation of certain proteins (such as the globulins) in the absence of salts.

Titration curves of gelatin between the limits of its two apparent isoelectric points are given and an estimation of the molecular weight of gelatin based on these data. The effect of sodium chloride up to 0.5 M on the viscosity and pH of 1 per cent solutions of pure gelatin is shown.

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RICKETS IN RATS.

XI. THE ALTERATION OF CALCIUM AND PHOSPHORUS METABOLISM OF NORMAL AND RICKETIC RATS PRODUCED BY IRRADIATED ERGOSTEROL.

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Minute amounts of irradiated ergosterol prevent and cure rickets in infants as well as in experimental animals (1–4). Large doses produce toxic effects (5–9). In such cases postmortem examination shows that calcium is deposited in the blood vessels, heart, stomach, lungs, kidneys, and muscles (7–9). A hypercalcemia is induced as well (9, 10). x-Ray studies and bone analyses show hypercalcification of the skeleton (9).

These observations present such striking evidence of a new and potent factor in calcium metabolism that a flood of questions arises. However, more data as to the effect of irradiated ergosterol must be collected before we shall be in a position to understand the mechanism of its action. How does it accomplish calcification, normal and pathological? Does irradiated ergosterol mobilize the minerals from the other tissues for bone deposition, or does it utilize the minerals in the food to better advantage? What is the normal and what is the optimal retention of calcium and phosphorus? Is the calcium and phosphorus retention solely dependent upon the vitamin content of the diet, or upon the mineral content, or are they interrelated?

Few have studied the influence of irradiated ergosterol upon the metabolism of calcium and phosphorus. Shohl, Bennett, and Weed (11) compared the curative action of cod liver oil and 0.01 mg. of irradiated ergosterol (100 times the minimum dose) when fed to ricketic rats. They found that cure occurred without marked

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increase in the calcium and phosphorus retention. Cartland, Spear, and Heyl (12) studied the effect of 0.0005 mg., 0.005 mg., and 0.05 mg. of irradiated ergosterol upon the calcium and phosphorus metabolism of normal rats. There was no change in retention with increased amounts of the vitamin. Hottinger (13) showed that very large amounts of irradiated ergosterol caused diminished balances of calcium and phosphorus in normal children and increased calcium balances in rickетic children. Light, Miller, and Frey (14) have recently reported that with excessive doses of irradiated ergosterol (100,000 times the minimum dose) given to normal rats diminished and even negative balances of calcium and phosphorus occurred and that the ash of the bones decreased markedly. The work to be reported here, which was done at the same time and without knowledge of their study, corroborates their findings and extends it in some particulars.

It was proposed to follow alterations of calcium and phosphorus metabolism in normal and rickетic rats with doses of irradiated ergosterol varying from small through optimal to toxic amounts. Studies were made (1) of the histological preparations of the soft tissues, (2) of the roentgenograms, (3) of the ash of the fat-free bones, and (4) of the intake and output of calcium and phosphorus in the urine and feces.

Normal Diet. Experiment I.

Plan.—Albino rats from the Wistar Institute experimental strain were bred in our laboratory. The parent stock animals were fed the modified Sherman Diet B (15).[1] The young were weaned at 23 days of age and continued on the same diet until 30 days of age when they averaged 49 gm. in weight.

In Experiment I, the rats were fed a normal diet with varying amounts of irradiated ergosterol. Twelve animals taken from two litters born on the same day were placed in metabolism cages (Hopkins type as modified by Gamble). Each cage held two rats, one from each litter. All were continued on the same diet and distilled water. Two of the twelve rats were kept as normal controls (without addition of vitamin D). In the other

[1] The modified Sherman Diet B consists of 32.9 per cent dried whole milk, 65.8 per cent ground whole wheat, 0.65 per cent sodium chloride, and 0.65 per cent calcium carbonate.

groups each rat was given daily doses of Vigantol[2] by mouth; 0.01, 0.10, 0.5, 1.0, and 2.0 mg. of irradiated ergosterol respectively. These amounts are 100 to 20,000 times the minimum curative dose (0.0001 mg.) and are equivalent, in vitamin D, to 0.5 to 100 gm. of cod liver oil. The Vigantol was so diluted with olive oil that each animal received approximately 0.1 cc. of oil daily.

Weights of the animals and the food intakes were recorded daily. The food spilled was recovered, weighed, and deducted from the gross food intakes to give the net food intakes. The urine and feces were collected separately. x-Rays were taken at intervals. The experiment was divided into three periods of 1 week each. The food and samples from the combined weekly output of urine and weekly output of stool were analyzed for calcium and phosphorus. At the end of the experiment, the animals, under light ether anesthesia, were killed by bleeding from the neck vessels. The rats were autopsied and portions of the ribs, heart and aorta, lungs, kidneys, stomach, and liver were taken for histological examination. The femurs of each animal were dissected for chemical analysis. The technique and analytical methods were similar to those used in previous experiments (16).

Progress.—The animals which received 0.01 mg. and 0.10 mg. of irradiated ergosterol appeared to be in better condition than the controls. They ate more food and grew more rapidly. Their coats were soft and glistening. Their flesh was firm to the touch. In contrast to these fine specimens, the animals which received 0.5 mg. of irradiated ergosterol ate less, grew less rapidly, and appeared much less robust. They were not in as good condition as the controls but were quite active and did not appear sick. The animals which received 1.0 and 2.0 mg. gained for the first 3 days, but thereafter lost weight. In order to accommodate our experimental procedure to this loss in weight, the first 3 days of metabolism Period 1 were studied separately from the last 4 days of

[2] The irradiated ergosterol employed in our experiments was a specially prepared Vigantol. A test of this preparation, by Miss D. N. Smith, showed that 0.0001 to 0.00025 mg. is sufficient, when fed to rats on the Steenbock diet, to protect against rickets. This material was kindly supplied by the Winthrop Chemical Company of New York through the courtesy of Dr. F. C. Waldecker.

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the same period (designated as Periods 1a and 1b). After 11 days the animals receiving 2 mg. of irradiated ergosterol lost so much weight and were so weak that it seemed doubtful whether they could live much longer; consequently they were killed. The group receiving 1.0 mg. was studied for a period of only 14 days for the same reason. The animals in these groups passed stools that were formed, but soft. Their eyes became sore and their noses red. The fur about the genitals was soiled and discolored.

Results.

Autopsy.—The animals receiving up to 0.10 mg. of irradiated ergosterol were normal and well nourished. Those given 0.5 mg. showed no gross abnormalities, but were less well nourished. Doses in concentrations of 1.0 mg. and 2.0 mg. cause changes typical of vitamin D poisoning; white spots and lesions in the heart, mottled patches on the surface of the kidney, and the cut surface showing a white line at the cortico-medullary junction. 1.0 mg. caused less severe changes than 2.0 mg. A detailed description of the changes observed under similar conditions is given by Shohl, Goldblatt, and Brown (9).

Histology.—The control rats fed on the modified Sherman Diet B showed no microscopic pathological changes. The animals fed 0.01 mg. of irradiated ergosterol in addition to the normal diet had slight parenchymatous degeneration of the kidneys and liver. This condition was more severe in the rats which received 0.10 mg. In those given 0.5 mg., calcification in small amounts occurred in the lining epithelium of some of the collecting tubules near the cortico-medullary junction of the kidneys. There was no calcification in any other organ. Tubular degeneration was quite severe in kidneys and liver, and a moderate number of mitoses were seen in the epithelial cells of these organs. In the rats which received 1.0 mg. of ergosterol, the calcification was more severe in the kidneys, affecting also the smaller vessels in the cortex as well as the tubular epithelium in both cortex and medulla. Calcification of the coronary vessels of the heart likewise occurred, and there were patches of calcification in the myocardium, some of which were surrounded by large and small mononuclear cells. There was a moderate deposit of calcium in the mucosa of the stomach. The two rats which

received 2.0 mg. of irradiated ergosterol had extensive calcification of the tubules and vessels of the kidney, in the vessels and muscle of the heart, mucosa and muscularis of the stomach, and interalveolar septa of the lungs. In the myocardium there were patches of necrosis and cellular infiltration with or without calcification of the myocardial fibers. Although the liver was not calcified, it showed severe degeneration and some mitoses of the liver cells.

x-Ray Study.—The roentgenograms of the controls showed normal bones throughout the experimental period. 2 days after the irradiated ergosterol was first administered, the films showed slight hypercalcification in all groups. A week later, this hypercalcification disappeared from the skeletons of the rats given 0.01 mg. In the other groups, the severity of the hypercalcification increased with increasing amounts of vitamin D up to 1.0 mg., but was less pronounced in the skeletons of the animals given 2.0 mg. Exposures taken during the 3rd week showed only slight hypercalcification in the bones of the animals given 0.1 mg. The severest hypercalcification obtained on any amount of irradiated ergosterol occurred in all the long bones of the animals given 0.5 mg. The hypercalcification extended even below the line of normal calcification.

Bone Analyses.—The results of the analyses are given in Table I. The ash, in per cent of the extracted weight, with 0.01 mg. of irradiated ergosterol was greater than that of the control; with 0.1 mg. it was maximal for the series; with 0.5 mg. it again approximately equaled that of the control. With greater doses the percentage and weight of the ash decreased quite markedly. The decrease of ash with the larger doses explains why the bones in this group are less dense roentgenographically than those in the 0.5 mg. group.

Metabolism of Calcium and Phosphorus.—Increasing doses of Vigantol caused an increase in the rate of growth to a maximum, followed by a sharp decline parallel to that observed in the bone ash. The controls on the average gained 19 gm. per week, those which received 0.01 mg. of irradiated ergosterol gained 21.6 gm., and those on 0.10 mg. gained 23.6 gm. Greater amounts of irradiated ergosterol markedly affected growth. The rats given 0.5 mg. averaged only 4.0 gm. gain while those with 1.0 and

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TABLE I.

Analyses of Bones of Rats Fed Varying Amounts of Irradiated Ergosterol.

Values per single femur.

Daily dosage of irradiated ergosterol.	No. of days given.	Final age of rat.	Weight.				Ash.	x-Ray findings.
			Wet.	Dry.	Extracted.	Ash.		
mg.		*days*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent of fat-free*	
Experiment I. Modified Sherman Diet B.								
0	0	52	327	169	164	85	51.9	Normal.
0	0	52	354	182	177	89	50.4	"
0.01	21	52	343	180	176	97	55.2	"
0.01	21	52	346	182	177	98	55.4	"
0.10	21	52	358	197	180	100	55.9	Hypercalcification +*
0.10	21	52	426	208	204	112	55.0	" +
0.5	21	52	276	146	139	65	46.7	" ++++
0.5	21	52	291	147	133	64	48.2	" ++++
1.0	14	45	245	109	101	41	40.9	" +++
2.0	11	42	213	93	84	33	39.6	" ++
Controls on modified Sherman Diet B.								
0	0	44	241	111	105	52	49.0	Normal.
0	0	56	301	155	147	83	56.5	"
0	0	65		176	146	84	57.4	"
Experiment II. Steenbock diet.								
0	0	65	258	115	101	40	39.6	Rickets.
0	0	65	447	206	182	77	42.7	"
0.01	14	65	266	122	110	47	42.6	Healing ++
0.01	14	65	418	214	189	90	47.7	" ++
0.10	14	65	303	141	136	62	45.6	" +++
0.10	14	65	326	161	152	70	46.2	" +++
0.5	14	65	236	111	101	45	45.6	" ++++
0.5	14	65	386	192	175	80	45.7	" ++++
1.0	14	65	300	138	121	55	45.3	Hypercalcification +
1.0	14	65	346	172	106	69	46.0	" +
2.0	6	57	222	96	95	39	41.0	" +
2.0	6	57	362	169	159	76	48.0	" +

* Scale of + to ++++, ++++ representing the highest degree.

2.0 mg. doses lost 2.5 and 7.0 gm. respectively. The average gain of 19 gm. for the control group compares very favorably with the gains reported by Sherman (17), 16.5 gm., and Boas (18), 16 gm., and the curve given by Donaldson (19), 15.6 gm., but is less than the growth obtained by Smith and Bing (15) on the modified Sherman Diet B, 28 gm.

The net food intakes paralleled the growth. The oil fed separately being disregarded, the food for the controls averaged 47.5 gm. per week. This amount was increased to 53.4 gm. with 0.01 mg. of irradiated ergosterol and to 57 gm. with 0.10 mg. The animals on the 0.5 mg. dose of the vitamin ate only 27 gm. and those on 1.0 and 2.0 mg. doses the still smaller totals of 21 and 17.3 gm. per week. The dry weight of the feces was roughly proportional to the net food intakes.

The figures for the metabolism of calcium and phosphorus are give in Tables II and III. There was a very definite shift of the calcium and phosphorus excretion from the feces to the urine following toxic doses of Vigantol. The animals which showed the change noticeably were those receiving 0.5 mg. or more of irradiated ergosterol. As may be seen by looking at Table II as a whole, there is an increase in the calcium in the urine from 20 per cent of the total excretion for the controls to 60 per cent for the groups given 1.0 and 2.0 mg. doses. Similarly the urinary phosphorus increases from 40 to 75 per cent of the total excretion.

The figures, shown in Table III, for the average calcium and phosphorus retained in the control group (175 mg. of Ca and 116 mg. of P), compare very favorably with those given in the literature for rats between 30 and 65 days of age. Boas (18) obtained 164 mg. of Ca and 113 mg. of P; Cartland, Spear, and Heyl (12), 134 mg. of Ca and 97 mg. of P; Light, Miller, and Frey (14), 196 mg. of Ca and 145 mg. of P. Schultzer (20) obtained 150 mg. of calcium and 92 mg. of phosphorus. Calculating from the data of Sherman (17, 21) on the calcium and phosphorus content of rats, we find that the retentions per week are 153 mg. of calcium and 106 mg. of phosphorus. The retentions obtained previously in our own laboratory were 195 mg. of Ca and 140 mg. of P (Diet E (22)). The amount of either element retained is seen to decrease slightly after the 1st week. For 0.01 mg. of irradiated ergosterol no change in the retention of calcium and

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phosphorus was observed. This is in agreement with Cartland, Spear, and Heyl (12). Our data show maximal values with 0.10 mg. of irradiated ergosterol. As much as 300 mg. of calcium and 135 mg. of phosphorus were retained in the 3rd week. These amounts represent 175 per cent of the normal calcium and 115 per

TABLE II.

Paths of Excretion on the Modified Sherman Diet B.

Figures are expressed in per cent of the total excretion.

Daily dose of irradiated ergosterol.	Period No.	Ca			P		
		Urine.	Feces.	Total excretion.	Urine.	Feces.	Total excretion.
mg.		*per cent*	*per cent*	*per cent of intake*	*per cent*	*per cent*	*per cent of intake*
0.00	1	25	75	20	43	57	35
	2	22	78	40	34	66	50
	3	10	90	42	37	63	51
0.01	1	8	92	25	34	66	38
	2	7	93	45	28	72	57
	3	5	95	37	33	67	50
0 10	1	38	62	21	51	49	39
	2	46	54	24	47	53	56
	3	26	74	23	29	71	56
0.5	1	17	83	18	39	51	25
	2	61	39	54	75	25	65
	3	50	50	54	70	30	68
1.0	1 a	62	38	26	56	44	42
	1 b	63	37	127	83	17	159
	2	63	37	54	73	27	70
2.0	1 a	61	39	41	70	30	86
	1 b	64	36	74	82	19	101
	2 a	57	43	81	75	25	93

cent of the normal phosphorus values. This is in agreement with the data of Light, Miller, and Frey (14) who found large increases of calcium and small increases of phosphorus with similar doses of vitamin D. With 1.0 mg. and 2.0 mg. of irradiated ergosterol vanishingly small or negative balances were obtained. This is

similar to the findings of Light, Miller, and Frey (14) with 10 mg. of irradiated ergosterol and irradiated yeast. Thus it is evident

TABLE III.

Calcium and Phosphorus Metabolism of Rats on Modified Sherman Diet B with Varying Doses of Irradiated Ergosterol.

Figures are expressed in terms of one rat per week.

Daily dosage of irradiated ergosterol.	Period No.	Ca						P						Ca:P retention.
		Intake.	Output.			Balance.		Intake.	Output.			Balance.		
			Urine.	Feces.	Total.				Urine.	Feces.	Total.			
mg.		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent of intake*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent of intake*	
0	1	238	13	35	48	190	80	188	29	37	66	122	65	1.56
0	2	276	10	101	111	165	60	218	37	72	109	109	50	1.51
0	3	298	12	115	127	171	58	235	44	75	119	116	49	1.48
0.01	1	236	10	49	59	177	75	186	25	47	72	114	62	1.56
0.01	2	312	8	131	139	173	55	246	39	100	139	107	43	1.62
0.01	3	348	7	122	129	219	63	275	46	93	139	136	50	1.61
0.10	1	272	21	36	57	215	79	215	42	41	83	132	61	1.63
0.10	2	311	34	40	74	237	76	246	67	72	139	107	44	2.22
0.10	3	390	25	65	90	300	77	308	67	106	173	135	44	2.22
0.5	1	169	5	25	30	139	82	134	13	21	34	100	75	1.39
0.5	2	145	48	30	78	67	46	114	56	18	74	40	35	1.68
0.5	3	148	39	38	77	71	46	117	55	23	78	39	33	1.82
1.0	1 a*	167	27	17	44	123	74	132	31	24	55	77	58	1.60
1.0	1 b†	80	64	38	102	−22		63	83	17	100	−37		
1.0	2	122	41	25	66	56	46	97	50	18	68	29	30	1.92
2.0	1 a*	113	28	18	46	67	59	90	54	23	77	13	14	5.15
2.0	1 b†	92	43	25	68	24	26	73	60	14	74	−1		
2.0	2 a†	96	45	34	78	18	19	76	53	18	71	5	7	2.57

* Although the figures are given in terms of one rat per week, the actual period studied was 3 days.

† Although the figures are given in terms of one rat per week, the actual period studied was 4 days.

that irradiated ergosterol in moderate doses over a period of time increases the calcium retention, but is without effect on the phosphorus retention; in large doses it causes diminished or even negative calcium and phosphorus balances.

The normal figure for the ratio of calcium to phosphorus retained, 1.52, is similar to that given by Boas (18), 1.46, Sherman (17, 21), 1.50, and Shohl, Bennett, and Weed (22), 1.41. With all doses the ratio is similar to that in the controls except for two groups. The ratio of those which received 2.0 mg. of irradiated ergosterol increases to 5.15. In this group the retentions are so small that the ratios have little significance. With 0.10 mg. of irradiated ergosterol the ratio is raised to 2.22. This rise indicates an excess calcium retention.

Rickets-Producing Diet. Experiment II.

In Experiment II, rats previously made ricketic were continued on a ricketogenic diet and in addition were fed varying amounts of irradiated ergosterol. Albino rats were bred and weaned as described above. Twelve animals (30 days of age) from two litters born on the same day were given the rickets-producing diet of Steenbock and Black[3] (23). After 3 weeks on this high calcium-low phosphorus diet, x-ray pictures showed marked rickets in all animals. Then the animals were placed in metabolism cages as in the previous experiment, two in a cage and one from each litter, and were continued on the deficient diet. Two rats were kept as rickets controls (without addition of irradiated ergosterol). The rats in the other groups were each given daily, by mouth, doses of irradiated ergosterol of 0.01, 0.10, 0.5, 1.0, and 2.0 mg. respectively. The experiment was continued for two periods of 1 week each. The studies of the calcium and phosphorus metabolism, the histological examinations, the x-ray pictures, and the bone analyses were conducted as in Experiment I.

Progress.—All the ricketic animals which received irradiated ergosterol behaved quite as usual on this diet. Although the animals grew at a diminished rate, they appeared active and well. The rats receiving 2.0 mg. of irradiated ergosterol lost con-

[3] The ricketogenic diet of Steenbock and Black consists of 76 per cent yellow corn, 20 per cent gluten flour, 3 per cent calcium carbonate, and 1 per cent sodium chloride.

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siderable weight and so were killed after the 6th day. They did not show the toxic symptoms noted in the normal animals which received the same dosage for 11 days.

Results.

Autopsy.—Rickets was present in the control rats as evidenced by the ricketic metaphyses of the enlarged and cupped costochondral junctions. All the other animals showed advanced healing. The kidneys were normal in all cases except for those receiving 2.0 mg. of irradiated ergosterol. Lesions in the kidneys were suggestive but not marked.

Histology.—Except for slight parenchymatous degeneration of the liver in the rats given 1.0 and 2.0 mg. of irradiated ergosterol, no microscopic pathological changes in the soft tissues were noted.

x-Ray Study.—Marked rickets was present in all animals at the beginning of the experiment as was shown by roentgenograms. At the end of the experiment, x-ray pictures of the controls showed severe rickets. Irradiated ergosterol, in amounts of 0.01 mg., partially healed the rickets; 0.10 mg. caused advanced healing; 0.50 mg. produced normal bones while 1.0 and 2.0 mg. caused an increased density at the epiphyseal end of the shaft of the long bones which was interpreted as hypercalcification.

Analyses of Bones.—The extracted femurs of the ricketic controls had an ash of 41 per cent (Table I) as compared with 31 per cent and 34 per cent reported previously (24). Although, after 5 weeks on the diet, the animals of the earlier experiments were losing weight and averaged 63 gm., these animals were still gaining weight on the ricketogenic diet and averaged 100 gm. We have some unpublished evidence to show that, in animals of the same age, regardless of the presence of a ricketic condition, the greater their weight, the greater is the bone ash in percentage of extracted femur. The evidence obtained from the x-ray, histology, blood serum, and calcium and phosphorus metabolism shows clearly that the control animals of this experiment had rickets. Addition of irradiated ergosterol to the diet increased the bone ash 10 to 15 per cent above that of the controls in all animals except those given 2.0 mg. The bone ash content of this group was the same as that of the controls, but 20 to 25 per cent below that of normal animals of the same age.

Metabolism of Calcium and Phosphorus.—The ricketic animals averaged 77 gm. when the Vigantol was first given to them. During the following 2 weeks the controls gained 5 gm. a week as compared to a loss of 2 gm obtained in previous experiments (25) (Table IV). No dose of Vigantol improved the growth of

TABLE IV.

Calcium and Phosphorus Metabolism of Rats on a Ricketogenic Diet with Varying Doses of Irradiated Ergosterol.

Figures are expressed in terms of one rat per week.

Daily dosage of irradiated ergosterol.	Period No.	Ca						P						Ca:P retention.
		Intake.	Output.			Balance.		Intake.	Output.			Balance.		
			Urine.	Feces.	Total.				Urine.	Feces.	Total.			
mg.		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent of intake*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent of intake*	
0	1	668	146	464	610	58	9	106	2	93	95	11	10	5.27
0	2	580	116	368	484	96	17	92	2	85	87	5	5	19.2
0.01	1	655	143	371	514	141	22	103	2	75	77	26	25	5.43
0.01	2	642	143	371	514	128	20	102	3	80	83	19	19	6.73
0.10	1	539	126	275	401	138	26	86	2	64	66	20	23	6.90
0.10	2	611	137	348	485	126	20	97	4	72	76	21	22	6.00
0.5	1	617	128	320	448	169	27	98	4	62	66	32	33	5.29
0.5	2	581	125	303	428	153	26	92	4	62	66	26	28	5.93
1.0	1	447	103	291	394	53	12	71	6	56	62	9	13	5.89
1.0	2	542	95	279	374	168	31	86	6	51	57	29	34	5.79
2.0	1	261	71	198	269	−8		41	30	30	60	−19		

the animals appreciably as compared to the controls. The toxic dose of 2 mg. of irradiated ergosterol produced a loss of 14 gm. per animal per week.

The net food intake of the ricketic controls was 50.7 gm. per rat per week. With 0.01 mg. the food intake was 52.7 gm.; 0.10 mg. gave 46.8 gm. intake; 0.5 mg., 48.7 gm.; 1.0 mg., 39.8 gm.:

and 2.0 mg., 24.5 gm. The dry weight of the feces was proportional to the food intake.

The paths of excretion were not changed by the addition of Vigantol and are similar to those in the controls which received the Steenbock diet, except in the group which received 2.0 mg. This group showed a definite shift of calcium and phosphorus from the feces to the urine, similar to that reported in Experiment I with smaller amounts.

The retentions of calcium and phosphorus calculated in mg. per rat per week are given in Table IV. The retentions on the Steenbock rickets-producing diet as determined previously (16, 25, 26) in our laboratory for rats between 30 and 65 days of age are 60 to 160 mg. of calcium and 20 to 45 mg. of phosphorus per week. The ricketic controls in this experiment showed even more deficient calcium and phosphorus retentions (58 to 96 mg. of Ca and 5 to 11 mg. of P). The ratio of calcium to phosphorus in the diet was larger (indicating a more severe ricketogenic diet) than in the former experiments (6.0 as compared to 4.0). The retentions depend upon the severity of the rickets, the rate of growth, and the salt content of the particular batch of diet. In all metabolism experiments upon ricketic rats, the calcium retentions are lower than in rats on a normal diet; those of phosphorus are still more deficient. Although irradiated ergosterol (0.5 to 1.0 mg.) caused the ricketic rat to retain more calcium than litter mates receiving no vitamin D, the actual amounts retained still fall within the range given above. The phosphorus retention, which is always so deficient in animals fed upon this diet, was not improved by the doses of vitamin D. Small negative balances were obtained with 2.0 mg. of irradiated ergosterol. Vitamin D does not alter the abnormal ratio of calcium to phosphorus retained. Upon the ricketogenic diet, the ratio of calcium to phosphorus retained is 3.0 or more. In the experiments described here the ratio was approximately 6.0. Even when 10,000 times the curative dose of irradiated ergosterol was administered, the relative amounts retained remain constant. Rickets was healed in these rats without materially altering the Ca and P retentions or ratios of retention. With the largest dose used, rickets was cured even though there were negative balances of calcium and phosphorus.

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DISCUSSION.

From the data presented it seems clear that rats fed upon an adequate diet are benefited in respect to growth and well-being when given moderate amounts of irradiated ergosterol and that huge amounts poison them, cause loss of weight and eventually death. Whether this holds true for any normal diet has not been determined. Other factors probably influencing the response of the animals to the vitamin are their environment, breeding, heredity, age, and weight, and the composition of the particular diet. The effect of the irradiated ergosterol is dependent upon the amount of the daily dose, the period during which it is administered, the potency of the particular preparation used, and the possible toxic impurities present. Other constituents of the diet may affect the toxicity. Light, Miller, and Frey (14) state that non-irradiated yeast counteracts the deleterious effects of irradiated ergosterol. Their rats fed upon a normal diet containing irradiated yeast, with 10 mg. of irradiated ergosterol added, survived for 2 weeks. In our laboratory, animals given 4.0 mg. of irradiated ergosterol in addition to the modified Sherman Diet B, died in 1 week. Provided the potency of the ergosterol was the same, it is necessary to conclude that the yeast protected their rats against the action of the vitamin.

The ricketic animal is much more resistant to toxic doses of vitamin D than the normal animal. Ricketic rats which received 2.0 mg. of irradiated ergosterol did not show as severe pathological changes after 6 days as did the rats on a normal diet given 2.0 mg. for 3 days (9). In children also, it has been shown that a dose of irradiated ergosterol, which is toxic for normal children, is beneficial for those with rickets (10, 13).

The question is naturally raised whether vitamin D affects the calcium and phosphorus retention by controlling the absorption or excretion of these elements. Much of the early literature on this subject has maintained that increased urinary excretion means better absorption from the gut. We have been unable to determine how much fecal calcium is unabsorbed (residual), or excreted, or secreted, and have therefore tentatively regarded the total as that excreted. The data indicate that with 0.01 mg. of irradiated ergosterol there is a slight diminution in the urinary calcium excretion, thus resulting in a slightly improved calcium

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retention. With increasing doses, there is a definite shift of calcium and phosphorus from the feces to the urine. This is in agreement with the findings of Holtz (27). However, the food intakes increased with 0.01 and 0.10 mg. of irradiated ergosterol and decreased with larger amounts. Therefore, the amount of calcium and phosphorus excreted, in per cent of that eaten was in a relatively constant proportion until toxic manifestations supervened, when the amount excreted in the urine increased. Our figures show increased retentions of calcium, both with diminished (*cf.* Experiment I with the 0.01 mg. dose) and increased (*cf.* Experiment II with the 0.10 mg. dose) urinary excretion. Therefore, from the amount in the feces and urine alone, as has been considered customary in the past, it is difficult if not impossible to draw any conclusions as to the amount absorbed or excreted.

Similar deductions may be drawn from the data on the bones. The analyses showed that large amounts of vitamin D resulted in a demineralization of the skeleton. We found also that the same large doses produced abnormally large urinary excretion of calcium and phosphorus. We therefore regard the increased excretion as due to a solution of the bone salts rather than to an increased absorption from the gut.

The data for calcium and phosphorus metabolism on a normal diet show normal retentions for small doses of vitamin D, increased retentions for moderate doses, and diminished or negative balances for toxic amounts. The effect of the vitamin is more marked upon the calcium than the phosphorus. With ricketogenic diets, parallel phenomena are observed.

The effect of the mineral constitution of the diet is also clearly indicated in the above experiments. On the normal diet approximately 3 times as much calcium and 5 times as much phosphorus were retained as on the ricketogenic diet. This holds true whether vitamin is administered or not. Therefore, although there is interdependence between the mineral and vitamin content of the diet, as Bethke, Steenbock, and Nelson (28) have pointed out, the absolute amounts retained and the ratio of retention are dependent, primarily, upon the salt constituents of the diet. The calcium and phosphorus must be present in the diet in sufficient amounts and in correct relationship to each other before

the rats can retain them in proper amounts and in correct relations for normal bone deposition, vitamin or no vitamin.

The effects of vitamin D on calcification as determined by x-ray and bone analyses indicate that moderate doses cause increased deposition at the epiphyses, and large doses, demineralization of the bones. The same effect was shown when the phenomenon was followed day by day (9). After a single dose of 4.0 mg. of irradiated ergosterol, the ash in mg. was raised 12 per cent. With the same dosage daily, this deposit disappeared on the 3rd day and thereafter the bones became progressively demineralized. However, when rickets was present, vitamin administration caused calcification at the epiphyses, even when the bones became demineralized. Vitamin D does not markedly affect the ratio of calcium and phosphorus retention. It is unable to alter deficient calcium and phosphorus retentions. However, in spite of the calcium and phosphorus metabolism, vitamin D causes calcification of the skeleton. This indicates that the action of vitamin D is to dissolve the lime salts from the shaft of the bones and deposit them in the growing end. The total mineral deposition is a resultant of these two reactions. Vitamin D insures the most efficient use of the minerals which are present in the body by regulating their intermediary metabolism.

SUMMARY.

The normal albino rat improves in weight and general well being with increased doses of irradiated ergosterol up to a maximum of 0.10 mg. per day. The bone ash becomes heavier and the calcium retention greater. With larger doses (0.5 to 2.0 mg.) toxic symptoms are characterized by a lessened bone ash, a shift of the calcium and phosphorus from the feces to the urine, and reduced or negative calcium and phosphorus balances. Pathological calcification results.

Although rickets is cured by irradiated ergosterol, the general well being of the animal is not greatly improved. Ricketic rats are more resistant to toxic amounts of irradiated ergosterol than normal animals. Irradiated ergosterol caused the calcium and phosphorus retentions to increase slightly over the control, but these remained far below normal. Negative balances were

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obtained with 2.0 mg. of irradiated ergosterol. Vitamin D did not alter the ratio of calcium and phosphorus retained.

Vitamin D controls calcification of the skeleton by dissolution and deposition of the bone salts. The ash content of the bones is a resultant of these two activities. The amount of calcium and phosphorus in the diet determines the retention of these elements. Vitamin D controls their intermediary metabolism.

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THE ISOLATION OF ADENOSINE FROM HUMAN URINE.

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The relatively wide distribution of adenosine phosphoric acid or adenine nucleotide in nature might lead one to suspect its presence in urine. It has been isolated from heart muscle (1), skeletal muscle (2), milk (3), blood (4), pancreas (5), and embryonic tissue (6), as well as from a number of plant sources. The relative ease with which it can usually be obtained from its natural sources led to an attempt to identify it among the many unidentified nitrogenous substances present in urine. As is often the case, the original purpose of the investigation was not attained. No trace of adenine nucleotide was found when the solution was treated with lead acetate, a procedure which usually precipitates nucleotides. However, nucleosides are precipitated by lead acetate in alkaline solution. The acid filtrate from the lead acetate precipitate was made alkaline with ammonia and a heavy precipitate was obtained from which adenosine was subsequently isolated, first as the picrate and finally as the free crystalline adenosine.

EXPERIMENTAL.

The urine used in this investigation was collected by approximately 60 students. 2 volumes of 40 liters each were used and the solutions combined near the end of the experiment. A procedure was followed which resembles the technique of Embden and Zimmerman (2) in the preparation of adenine nucleotide from muscle.

A large quantity of the phosphates was removed at the outset of the experiment by making the solution alkaline with a suspension of calcium oxide in water. The supernatant liquid was siphoned off from the precipitated phosphates and 100 cc. of 10 per cent

copper sulfate solution were added for every liter of solution, the solution being kept alkaline to litmus. This procedure precipitates nucleotides and nucleosides. The precipitate obtained was removed by centrifuging, washed with lime water, suspended in hot water, and treated with hydrogen sulfide to remove the copper. After filtration the solution was aerated to remove the hydrogen sulfide. The solution was made alkaline with calcium oxide and the precipitation with copper sulfate was repeated, the solution being kept alkaline as above.

After removal of the hydrogen sulfide, the solution was acidified with acetic acid, 5 cc. of a 25 per cent solution of lead acetate were added for every liter of urine used, and the solution was allowed to stand overnight. If nucleotides had been present they would have precipitated at this point, but only a very small amount of precipitate appeared, which gave negative pentose and purine tests. When the supernatant fluid after decantation was made alkaline with ammonia a heavy precipitate was obtained. The precipitate was centrifuged off, washed with water, finally suspended in hot water, and the lead removed with hydrogen sulfide. The hydrogen sulfide was removed by aeration and the precipitation with lead acetate in alkaline solution repeated. After the removal of the lead and the subsequent removal of hydrogen sulfide, the solution was decolorized with norit, then concentrated to a thin syrup, and a saturated solution of picric acid in alcohol was added until a small amount of the filtered solution gave no further precipitate. The precipitate, when recrystallized from hot water, had a melting point of 182° (uncorrected) and gave the analytical values reported below. The yield was about 300 mg. from 80 liters of urine. When a mixed melting point of the above picrate and adenosine picrate from yeast nucleic acid was made, the melting point did not change.

Samples weighing from 5 to 6 mg. gave the following analytical values of the picrate for carbon, hydrogen, and nitrogen, the micro-Pregl method being used for carbon and hydrogen, and the micro-Dumas-Pregl procedure for nitrogen (7).

I. 5.180 mg.: 7.336 mg. CO_2 and 1.667 mg. H_2O.
II. 6.731 " : 9.524 " " " 1.986 " "
III. 5.391 " : 1.081 cc. N at 26° and 746 mm.
IV. 5.821 " : 1.166 " " " 26° " 746 "

	Calculated for $C_{10}H_{12}N_5O_8$-$C_6H_2(OH)$-$(NO_2)_3$.	Found.			
		I.	II.	III.	IV.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
C	38.71	38.61	38.50		
H	3.22	3.57	3.28		
N	22.58			22.49	22.46

Free adenosine was prepared by dissolving the picrate in dilute sulfuric acid and extracting the picric acid with ether. The sulfuric acid was removed by lead carbonate, the final traces of lead by means of hydrogen sulfide, and the excess hydrogen sulfide by aeration. The solution was decolorized with norit and concentrated to a very small volume from which a few mg. of precipitate were obtained which gave the following analytical values.

Micro-Dumas-Pregl for nitrogen (7).

I. 4.732 mg.: 1.002 cc. N at 25° and 741 mm.
II. 3.876 " : 0.819 " " " 25° " 741 "

Required for $C_{10}H_{13}N_5O_4 \cdot 1\frac{1}{2}H_2O$..... N 23.81.
Found. I..... " 23.66.
II..... " 23.61.

DISCUSSION.

The occurrence of adenosine in urine is of interest for several reasons. Although nucleotides have been isolated from various sources and although enzymes for hydrolysis of nucleotides to nucleosides are present in animal and plant tissues, only two free naturally occurring nucleosides have been previously reported in the literature. One of these is the substance which Schulze and Bosshard (8) obtained in 1886 from plant tissues and called vermine and which Schulze (9) found to be guanine pentoside. Later Schulze and Trier (10) showed that it was identical with a sample of guanosine prepared by Levene. The other nucleoside is the uric acid-pentose isolated from blood by Benedict (11). This nucleoside is probably not a product formed by the union of uric acid and pentose but rather an oxidation product from adenine nucleotide through the intermediary product hypoxanthine nucleotide (inosinic acid) to uric acid nucleotide and then loss of phosphoric acid by hydrolysis, or it may be an oxidation product

of adenosine or inosine, which are obtained from adenine nucleotide and hypoxanthine nucleotide respectively. In view of the presence of adenosine in urine the latter is the more probable mechanism although inosine has never been found in animal tissues. It should be kept clearly in mind that the isolation reported in this paper was from a mixed urine and that the yield was very small. It might easily have had its origin in the urine of a few individuals and, therefore, may not be of common occurrence.

Of further interest is the relationship that the adenosine isolated in the present investigation may have to the adenine nucleotide which was isolated from heart muscle by Szent-Györgyi and Drury (1) and which had, if injected intravenously, a definite action on the heart. The part of the molecule responsible for this action was the adenosine compound. Furthermore Doyon and Vial (12), have reported the isolation of a very active anticoagulating substance by extraction of mesenteric ganglions which they believed was a nucleoside. However, they did not definitely identify the compound.

SUMMARY.

A crystalline picrate was isolated from urine which had the same analytical values and melting point as adenosine picrate obtained from yeast nucleic acid. A mixed melting point of the two compounds was the same as that of each of the individual substances. A crystalline compound was obtained after removal of the picric acid which had the same nitrogen content of adenosine obtained from yeast nucleic acid.

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THE EFFECT OF IRRADIATED ERGOSTEROL ON THYROPARATHYROIDECTOMIZED DOGS.*

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In a previous publication (1) it was shown that the feeding of cod liver oil to dogs for a period of 2 weeks before the removal of the thyroid and parathyroid glands greatly influenced the results of such an operation. The calcium of the blood serum fell as usual after parathyroidectomy, but the animals either did not develop tetany or the attacks were slight. The length of life of the treated dogs was greatly increased over that of the controls. Feeding of the oil after the operation was without apparent effect.

More recently papers have been published by Brougher (2) and by Wade (3) in which they have reported somewhat similar results. In not all cases was tetany prevented, but it was greatly delayed and the attacks were rather mild. Greenwald and Gross (4) on the other hand were unable to substantiate these findings.

Since preoperative treatment with cod liver oil appeared to exert a definite influence on the results following thyroparathyroidectomy, it was thought advisable to study the effect of activated ergosterol. Several papers have already appeared in which are reported the results of treatment with irradiated ergosterol after the removal of the parathyroids. Urechia and Popoviciu (5) administered it to six dogs subcutaneously and by mouth. Four of the dogs died in tetany. The other two recovered from the tetany and lived for about 2 months, during which time there was a slight rise in the concentration of serum calcium. Brougher (6) claims that the giving of activated ergosterol in conjunction

* A report of this investigation was given before the Physiological Society of Philadelphia on November 18, 1929, *Am. J. Med. Sc.*, **179**, 144 (1930).

with milk greatly decreased the severity of the attacks of tetany, although the calcium concentration of the blood was not increased. Several of the dogs recovered from the tetany entirely, and in time the level of serum calcium returned to normal. Hess and associates (7) were unable to raise the serum calcium of monkeys and dogs, from which the parathyroids had been removed, by giving large doses of irradiated ergosterol. Greenwald and Gross (4), using dogs, report similar results.

EXPERIMENTAL.

Dogs were used as the experimental animals. A modification of the Karr-Cowgill (8) diet was employed as a basal ration. It is composed as follows:

	per cent
Casein	37.6
Sucrose	32.9
Lard	20.0
Salt mixture	2.0
Yeast	5.0
Agar	2.5

The Cowgill salt mixture was used with the slight modification of omitting the $CaHPO_4$ and substituting an equimolecular amount of $Na_2HPO_4 \cdot 12H_2O$. No bone ash was fed. Although no calcium salt was added to this diet it contained about 0.25 per cent calcium. The ration for the most part was fed at a level of 16 gm. per kilo of body weight. There were necessarily some exceptions in individual cases. A 1 per cent solution of irradiated ergosterol[1] was used, which in most experiments was given directly to the animal from a pipette. The surgical operations[2] consisted of complete removal of the thyroparathyroid apparatus. Serum calcium was determined by the method of Clark and Collip (9). In order to detect any change that might occur in blood concentration, hemoglobin determinations were made throughout these experiments by the method of Newcomer (10). The results are expressed as per cent of normal, the Haldane scale, which is based

[1] The irradiated ergosterol was kindly furnished by the Winthrop Chemical Company of New York.

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TABLE I.

Effect of Administration of Cottonseed Oil and of Irradiated Ergosterol upon Prevention of Tetany, Duration of Life, and Concentration of Serum Calcium

TABLE I.

Effect of Administration of Cottonseed Oil and of Irradiated Ergosterol upon Prevention of Tetany, Duration of Life, and Concentration of Serum Calcium.

Dog No.	Treatment.		Days after operation.		Serum Ca (mg. per 100 cc. serum).	
	Before operation.	After operation	Before tetany.	Before death.	At operation.	After operation.
33	Basal ration 1 + 20 cc. cottonseed oil for 12 days.	Basal Ration 1.	2	9+	11.65	(7)* 4.96
34	" "	" " 1.	4	4	12.70	(2) 8.56
39	Basal Ration 1 + 20 cc. cottonseed oil for 14 days.	" " 1.	3	3+	11.20	
49	Basal Ration 2 + 20 cc. cottonseed oil for 14 days.	" " 2.	4	9+	10.81	(3) 7.22
50	" "	" " 2.	3	11+	9.05	(3) 6.06
61	Basal Ration 2 for 12 days.	" " 2.	4	4+	10.52	(2) 5.99 (4) 4.63
66	" " 2 " 8 "	" " 2.	No tetany observed.	3	12.75	
57	" " 2 + 4 mg. ergosterol daily for 14 days.	" " 2.	" "	Experiment discontinued after 111 days.		(8) 11.59 (12) 7.24 (34) 5.37 (41) 5.22 (49) 10.82 (59) 10.52
59	Basal Ration 2 + 4 mg. ergosterol daily for 16 days.	" " 2.	12	15+		(3) 8.16 (13) 4.81
60	" "	" " 2.	8	13		(3) 8.27 (13) 5.02
63	Basal Ration 2 + 10 mg. ergosterol daily for 14 days.	" " 2.	No tetany observed.	Experiment discontinued after 93 days.	16.75	(17) 10.49 (31) 9.85 (39) 7.09 (44) 6.19 (50) 5.89 (93) 7.89
77	Basal Ration 2 + 10 mg. ergosterol daily for 16 days.	" " 2.	38	44	16.0	(13) 10.70 (38) 6.04

* The figures in parentheses indicate the number of days after operation when the blood sample was taken.

on a normal of 13.8 gm. of hemoglobin per 100 cc. of blood, being used. The concentration of the inorganic phosphorus of the serum, which was followed only during the latter half of these studies, was determined by the method of Fiske and Subbarow (11).

During the course of the investigation several control animals were studied. Some of these received the basal ration for a period previous to the removal of the glands, and others were given the ration only after the surgical operation was performed.

To determine whether the effects previously obtained with cod liver oil were due to the caloric content of the fat, cottonseed oil, which is known to have but slight antirachitic properties, was given to several dogs for a period of about 2 weeks before the removal of the glands. Three of these dogs (Dogs 33, 34, and 39) received the same basal ration as was used in the work previously reported (Ration 1), and two (Dogs 49 and 50) were given the basal ration described above (Ration 2). The results, summarized in Table I, show that this oil is without effect. These findings negate the objection raised by Greenwald and Gross (4) who suggested that the results previously reported might have been due to the decrease in consumption of basal ration caused by the additional calories of the cod liver oil.

Two dogs (Dogs 61 and 66) were given the basal ration without any oil. These animals also died within a few days after the removal of the glands. Dog 61 was observed in tetany shortly before death.

In marked contrast to these results are those obtained by feeding irradiated ergosterol (Table I). Dogs 57, 59, and 60 were given 4 mg. of ergosterol daily for about 2 weeks previous to the operation. At no time did Dog 57 show tetany although the concentration of calcium fell as low as 5.22 mg. per 100 cc. of serum. The experiment was discontinued on the 111th day following the operation at which time the calcium had returned to normal. The gradual increase in the level of the serum calcium after a very low concentration has been reached was previously reported by Brougher (2). Throughout the entire period this animal appeared well and acted in a perfectly normal manner. Dogs 59 and 60 both showed tetany and died in a rather short time after the operation. The tetany, however, was somewhat delayed

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and not very severe, and each animal lived slightly longer than any of the controls.

Dogs 63 and 77 were given 10 mg. of ergosterol daily for about 2 weeks before extirpating the glands. Both these animals showed hypercalcemia at the time of the operation. The concentration of calcium of Dog 63 fell below the tetanic level without the animal showing any signs of tetany. There followed a slight rise in calcium level, but it was still below normal when the experiment was discontinued on the 93rd day following the operation. Dog 77 was in slight tetany on the 38th and died on the 44th day after the removal of the glands. The tetany consisted of nothing more than a few muscular twitchings.

The results reported here on ergosterol in conjunction with those previously reported on cod liver oil show conclusively that preoperative treatment with the antirachitic factor markedly influences the results obtained by removing the parathyroid glands from dogs. The length of life is greatly increased, and tetany is either entirely prevented or its onset is considerably delayed. The attacks of tetany, if occurring at all, are usually very slight. The concentration of serum calcium of the treated animals falls more slowly than in the controls. This is particularly true of the animals treated with irradiated ergosterol. The most striking thing, however, is the fact that many of the animals may not develop tetany though the level of blood calcium be low. In some cases the animals completely recovered after the removal of the glands, and in others the dogs died although they had experienced little or no tetany. The recovery can best be explained by hypertrophy of residual parathyroid tissue. However, there are no data to substantiate this theory. The cause of death in the absence of severe tetany is likewise difficult to explain. It probably means that death following parathyroidectomy is frequently due to some cause other than tetany.

After obtaining the results given above a study was made of the postoperative treatment with irradiated ergosterol.

Several investigators (7, 12) have recently shown that overdoses of irradiated ergosterol cause a hypercalcemia in a normal animal. However, Hess and associates (7) and Greenwald and Gross (4) were unable to produce any noticeable rise in the concentration of calcium in the serum of parathyroidectomized ani-

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TABLE II.

Effect of Irradiated Ergosterol Administered before and after Thyroparathyroidectomy.

Date.	Diet and treatment.	Ca per 100 cc. serum.	P per 100 cc. serum.	Hb	Remarks.
			Dog 64. Female; weight, 14.1 kilos.		
1929		*mg.*	*mg.*	*per cent*	
Feb. 14	Began basal ration + 10 mg. ergosterol.				Condition good.
" 16		10.43		101	Eating well.
Mar. 1		12.63		103	" "
" 2	Glands removed. Discontinued ergosterol.				
" 5	Basal ration.	10.70		121	Not eating.
" 8	" "	9.34		115	Eating irregularly.
" 13	" "	8.20		117	" "
" 15	" "				Tetany.
" 16	" "	5.37		108	Quiet.
" 16–22	50 mg. ergosterol daily.				
" 18		20.10			Eating well.
" 20		14.55		108	Not eating.
" 23	Discontinued ergosterol.	13.05		108	" "
" 29	Basal ration.	13.65		110	" "
Apr. 12	" "	12.58		103	Eating well.
May 10	" "	8.40		103	" "
" 17	Experiment discontinued.	8.96		105	
			Dog 67. Male; weight, 13.4 kilos.		
Mar. 19	Began basal ration.				Eating well.
" 29	Basal ration.	9.83		117	" "
Apr. 8	Began 10 mg. ergosterol daily.				" "
" 12		11.37			" "

TABLE II—*Continued.*

Date.	Diet and treatment.	Ca per 100 cc. serum.	P per 100 cc. serum.	Hb	Remarks.
Dog 67. Male; weight, 13.4 kilos—*Concluded.*					
1923		*mg.*	*mg.*	*per cent*	
Apr. 19	Glands removed. Discontinued ergosterol.	15.43	5.2	105	Eating well.
" 22	Basal ration.	12.55	3.9	108	Condition good.
May 1	" "	9.07	5.7	100	" "
" 3	" "	8.87	6.0	100	" "
" 10	" "	6.01	5.5	100	Tetany.
" 10–16	50 mg. ergosterol daily.				
" 13		10.37	10.9	98	No tetany.
" 17	Discontinued ergosterol.	15.85	5.8	100	Very active.
" 24	Basal ration.	17.75	4.3	100	Not eating.
" 31	" "	16.52	4.8	100	" "
June 7	" "	14.86	6.4	100	" "
" 14	" "	14.00	4.9	113	" "
" 21	" "	12.12	4.3	96	Eating.
" 28	" "	12.71	5.2	108	Not eating.
July 19	" "	13.72	5.5	105	Eating irregularly.
Nov. 7	Experiment discontinued.	10.40	5.1	89	
Dog 69. Female; weight, 17.5 kilos.					
Apr. 25	Began basal ration + 20 mg. ergosterol daily.	10.80	4.3	100	Condition good.
May 1	Glands removed. Discontinued ergosterol.	14.60	5.7	108	" "
" 3	Basal ration.	17.75	4.8	108	Not eating.
" 10	" "	15.05	4.5	109	" "
" 17	" "	14.48	4.0	100	" "
" 24	" "	10.15	6.2	83	Eating, condition good.

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TABLE II—*Concluded.*

Date.	Diet and treatment.	Ca per 100 cc. serum.	P per 100 cc. serum.	Hb	Remarks.
Dog 69. Female; weight, 17.5 kilos—*Concluded.*					
1929		*mg.*	*mg.*	*per cent*	
May 31	Basal ration.	8.48	7.3	79	Eating, condition good.
June 7	" "	8.30	7.1	79	" " "
" 14	" "	7.75	6.3	88	" " "
" 24	" "	7.34	6.6	86	" " "
" 28–30	50 mg. ergosterol daily.	7.73	6.5	99	" " "
July 1	Discontinued ergosterol.	15.75	6.1	108	" " "
" 10	Basal ration.	13.62	5.5	122	" " "
" 19	" "	12.38	5.9	103	" " "
Sept. 9	" "	7.70	6.5		" " "
" 30	" "	7.02	7.6	73	" " "
Nov. 7	" "	6.37	8.0	97	" " "
" 21	" "	5.06	6.9	91	Exercise, no tetany.
" 27	" "	5.06	8.8	99	Tetany.
" 27–Dec. 2	50 mg. ergosterol daily.				
Nov. 30		9.15	6.1	92	No tetany, condition good.
Dec. 3	Discontinued ergosterol.	16.16	5.6	94	Not eating, " "
" 9	Basal ration.	18.50	5.2	112	" " " "
" 22	" "				Dead.

mals. This led Hess and coworkers to postulate that the antirachitic factor acts by stimulating the parathyroid glands. This is in line with the idea expressed by Block and Faber (13) several years ago. They believed that ultra-violet light acts in preventing tetany by stimulating the parathyroids to greater activity.

Although no beneficial effects were obtained by postoperative treatment with cod liver oil, it cannot be concluded that the same would be true for irradiated ergosterol because the latter is much more active as an antirachitic agent. Any postoperative treatment must of necessity act quickly if it is to be beneficial to an animal that would otherwise die in a few days. On the other hand

if the duration of life is considerably prolonged by preoperative treatment the chances for beneficial postoperative treatment are increased. With this in mind three dogs (Dogs 64, 67, and 69) were given irradiated ergosterol for varying lengths of time before the removal of the glands. The feeding of the ergosterol was discontinued at the time of the operation. After the concentration of calcium had fallen to a rather low level large doses of ergosterol were again given. The results are summarized in Table II. All three dogs showed hypercalcemia at the time of the removal of the glands, following which there was a gradual fall of calcium until low levels were reached.

The concentration of calcium in the serum of Dog 64 dropped to 5.37 mg. per 100 cc. on the 15th day following the operation. On the previous day this animal experienced an attack of tetany. In 2 days time the daily administration of 50 mg. of irradiated ergosterol increased the calcium level from 5.37 to 20.10 mg. This was followed by a rather abrupt drop in calcium concentration to 14.55 mg. at which point the ergosterol was discontinued. The level of calcium then fell gradually until it reached 8.96 mg. on the 77th day after operation when the experiment was terminated.

The calcium concentration of the serum of Dog 67 fell from 15.43 mg. at the time of operation to 6.01 mg. 21 days later. Slight tetany was noted on this date. 50 mg. of ergosterol were given daily for 7 days and the level of calcium rose to 15.85 mg. Although the ergosterol was discontinued at this time the calcium concentration continued to rise and 7 days later was 17.75 mg. There then followed a prolonged and gradual drop in calcium concentration to 10.4 mg. on the 202nd day after the removal of the glands. The experiment was terminated at this time.

The results obtained from Dog 69 are of special interest. After the removal of the glands the fall of calcium was very slow. By the 56th day after the operation the calcium had decreased from 17.75 to 7.73 mg. The daily administration of 50 mg. of ergosterol increased the calcium level to 15.75 mg. in 3 days. The feeding of ergosterol was then discontinued and the concentration of calcium fell for several months. It finally reached the low level of 5.06 mg. on the 203rd day after extirpation of the glands. 2 weeks previous the calcium was 6.37 mg. During this

entire period the dog appeared perfectly well in every respect. It was active, ate well, maintained its weight, and showed no sign of tetany.

On the 209th day tremors were noticed while a sample of blood was being taken. Later in the day the dog was in typical tetany. The concentration of calcium at this time was exactly the same as it was 6 days previous, at which time the animal showed no indication of tetany. This again shows that there must be some factor or factors in addition to the concentration of calcium which influence the development of tetany. If tetany was not present on the 203rd day after the removal of the glands, because of an adjustment on the part of the organism to the low concentration of serum calcium, as Greenwald and Gross (4) suggest, it should not have appeared 6 days later with the concentration of calcium remaining the same. At the time of the appearance of tetany the daily administration of 50 mg. of ergosterol was again started. 3 days later the serum calcium had risen to 9.15 mg. and by the 6th day after the beginning of ergosterol feeding it had attained the level of 16.16 mg. The administration of ergosterol was discontinued at this point. 6 days later the serum calcium was 18.50 mg. per 100 cc. at which time the animal was active and appeared perfectly well but would not eat. 13 days after the last sample of blood was taken the dog was found dead. At autopsy no definite signs of abnormal calcification could be detected. There were, however, several non-calcified nodules on the interior surface of the left ventricle of the heart. They were probably the result of the cardiac punctures performed at the time of taking blood samples. Death was possibly caused by an embolus arising from one of these nodules. In 7 months time the parathyroid tissue (if any be present) had not hypertrophied sufficiently to prevent the development of a marked hypocalcemia and finally the onset of tetany. The administration of ergosterol resulted in an immediate improvement. The data obtained from this animal, which had a severe parathyroid deficiency if not a complete absence, demonstrate rather strikingly that it is not only possible to keep parathyroidectomized dogs alive for several months but also in perfect condition symptomatically by the administration of irradiated ergosterol.

Having obtained such striking results with the three dogs

TABLE III.

Effect of Irradiated Ergosterol Administered after Thyroparathyroidectomy.

Date.	Diet and treatment.	Ca per 100 cc. serum.	P per 100 cc. serum.	Hb	Remarks.
	Dog 71. Male; weight, 6.7 kilos.				
1929		*mg.*	*mg.*	*per cent*	
June 3	Basal ration.	11.0	7.4		Condition good.
" 4	Glands removed.				" "
" 6	100 mg. ergosterol.	5.78	8.4	101	Tetany.
" 7	50 " " + 150 cc. milk.				" vomited.
" 8	50 mg. ergosterol + 100 cc. milk.				Muscle twitchings.
" 9	" "				Tremors, vomited.
" 10	" "				" "
" 11	" "	5.78	7.6	75	" "
" 12	50 mg. ergosterol + 75 cc. milk.				" "
" 13	" "				No tremors, vomited.
" 14					Dead.
	Dog 72. Male; weight, 10.6 kilos.				
June 3	Basal ration.	10.45	7.2		Condition good.
" 4	Glands removed.				" "
" 6	100 mg. ergosterol.	5.45	7.0	99	Tetany.
" 7	50 " " + 250 cc. milk.				"
" 8	" "				Condition good.
" 9	50 mg. ergosterol + 200 cc. milk.				" "
" 10	" "				" "
" 11	Basal ration + 50 mg. ergosterol.	8.10	9.7	75	" "
" 12	" "				" "
" 13	" "				" "
" 14	Discontinued ergosterol.	14.95	7.6	68	" "
" 18	Basal ration.	17.60	7.1	83	Not eating.
" 21	" "	15.60	7.1	83	Eating, condition good.
" 28	" "	10.62	5.0	85	
July 6	" "				Tetany.
" 8		7.24	7.13	99	Dead, blood taken after death.

TABLE III—*Continued.*

Date.	Diet and treatment.	Ca per 100 cc. serum.	P per 100 cc. serum.	Hb	Remarks.
	Dog 73. Male; weight, 12.9 kilos.				
1929		*mg.*	*mg.*	*per cent*	
July 20	Began basal ration.				
" 22	Glands removed.	10.66	7.7	88	Condition good.
" 23	Basal ration.	7.28	9.2	82	" "
" 24	100 mg. ergosterol + 200 cc. milk.	5.85	10.7		Tetany.
" 25–Aug. 1	50 mg. ergosterol daily.				Active. No tetany.
July 26		5.75	10.8	80	
" 29		6.69	10.4	68	
" 31		9.76	9.1	74	
Aug. 2	Discontinued ergosterol.	14.15	6.8	75	
" 6	Basal ration.	32.80	16.3	75	Not eating.
" 7	" "	47.27	19.1	80	" "
" 11	" "	22.85		70	" "
" 14	" "	15.25			" "
" 18	" "	13.26		70	" "
" 25	" "	9.90	6.4	78	Eating well.
Sept. 1	" "	8.48	4.5	78	
" 4	" "	7.30	6.6		
" 7	" "				Tetany.
" 8	" "	8.3	7.3		"
" 13	" "				
" 17	" "	8.3	6.5		Died during taking of blood.
	Dog 75. Female; weight, 7.7 kilos.				
Aug. 5	Began basal ration.				Condition good.
" 7	Glands removed.	11.61	5.1	104	Eating well.
" 8	Basal ration.	8.21	7.1		Condition good.
" 9		7.16	7.1		Quiet.
" 9–23	50 mg. ergosterol daily.				
" 11		15.78		100	Eating well.
" 14		12.11			Active.
" 18		19.56		95	Not eating.

TABLE III—*Concluded.*

Date.	Diet and treatment.	Ca per 100 cc. serum.	P per 100 cc. serum.	Hb	Remarks.
Dog 75. Female; weight, 7.7 kilos—*Concluded.*					
1929		*mg.*	*mg.*	*per cent*	
Aug. 24	Discontinued ergosterol.				Not eating.
" 25	Basal ration.	21.60	5.7	97	" "
Sept. 1	" "	18.85	3.5	95	" "
" 8	" "	16.90	3.8	100	" "
" 18	" "	13.26	3.4	83	Eating well.
" 30	" "	10.53	4.5	70	" "
Nov. 27	" "	9.52	6.0	98	" "

which were given preoperative and postoperative treatment, it was thought advisable to study the effect of treatment only after the glands had been removed. For this purpose the thyroids and parathyroids were removed from six dogs (Dogs 70 to 75) that had had no preoperative treatment of any kind. Dogs 70 and 74 were kept as controls. Both of these animals died during the second night following the operation.

Treatment of the other animals (Table III) was delayed until analyses showed a low concentration of serum calcium. Three of the animals experienced severe tetany before any ergosterol was given. Treatment was started at this time, instead of immediately following the operation, in order to demonstrate the removal of at least sufficient amounts of the parathyroids to produce the usual results; *i.e.*, a rapid fall in concentration of serum calcium accompanied by tetany.

2 days after the glands were removed Dog 71 was in tetany with a calcium level of 5.78 mg. At this time milk and ergosterol were given in amounts as shown in Table III. These materials were given daily, by stomach tube, but the dog vomited immediately after each feeding. The dog died on the 9th day after operation. On the 7th day the calcium level was 5.78 mg.

The results obtained from Dog 72 are shown in Table III. This dog, with a calcium concentration of 5.45 mg., was in tetany

on the 2nd day following the removal of the glands. 100 mg. of ergosterol were given with no vomiting, and on the following day 50 mg. of ergosterol and 250 cc. of milk were administered. 24 hours later the condition of the dog was greatly improved and the treatment with milk and ergosterol was continued. The milk was given to aid in carrying the animal through the period of attacks of violent tetany, and it was probably an important factor in improving the condition of the animal. However, the improvement was followed by a marked rise in concentration of serum calcium which cannot be attributed to the milk alone. The milk was discontinued after the 4th day. The calcium continued to rise from 5.45 mg. at the beginning of ergosterol feeding to 14.95 mg. in 7 days, when the administration of ergosterol was discontinued, but the calcium level continued to rise and 4 days later it was 17.6 mg. This was followed by a drop in calcium concentration to 7.24 on the 65th day after operation. The dog died on this date and the blood sample was taken after death. 2 days previously the dog was observed in tetany.

After the removal of the parathyroids from Dog 73 there was an immediate drop in concentration of serum calcium accompanied by tetany. Ergosterol was administered as in the preceding experiment (Table III). Milk was given on 1 day only. For a few days there was little change in the calcium level, but there was soon a very sharp rise to an extremely high value. On the 9th day after the beginning of ergosterol administration, the concentration of calcium was 14.15 mg. 4 days later the calcium value was 32.80 mg. and on the following day it had risen to the extreme height of 47.27 mg. per 100 cc. of serum. Although duplicate analyses checked, a third determination was made on the day that the sample containing 32.80 mg. of calcium was analyzed. A different solution of potassium permanganate was used for this titration, and this determination checked the others within 1 mg. per 100 cc. In addition, the blood from a normal dog was analyzed simultaneously with the sample which gave 47.27 mg., and the calcium content of this serum was found to be 11.61 mg. By simply looking at the precipitate of calcium oxalate it was obvious that there was an exceptionally large amount present. Further confirmation for the correctness of the calcium analyses is found in the concentration of inorganic phosphorus. Beginning

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with Dog 67 the inorganic phosphorus of the blood of these animals was determined. In most cases there is apparently no relation between the concentrations of calcium and phosphorus as shown by the accompanying tables. Several investigators (14) have previously reported that irradiated ergosterol has no constant effect on the phosphorus level as it does on calcium. It has, however, been demonstrated that it is possible to raise the level of inorganic phosphorus by injecting calcium salts into the blood stream (15). The results with Dog 73 would indicate that the increase of serum phosphorus in this experiment was an accompaniment of the very high calcium. In the other experiments the calcium was not increased sufficiently to influence to any marked degree the level of phosphorus. Following this unusually high concentration of calcium there was a rapid fall, and on the 47th day after operation the dog was again in tetany. On the following day, when the dog showed no tetany, the concentration of calcium was 8.3 mg. For the next 10 days the dog was in slight tetany from time to time, and died while a sample of blood was being taken on the 57th day after the removal of the glands.

The data obtained from Dog 75 (Table III) are quite similar to those previously described. No milk was given to this animal. The dog is still on experiment. Irradiated ergosterol was given to seven dogs after the removal of the parathyroid glands. In six of these seven a marked hypercalcemia was produced. In every case the concentration of serum calcium was considerably below normal when ergosterol feeding was started. The one case of failure was in all probability due to the gastrointestinal upset causing the dog to vomit the ergosterol and milk which were given. No attempt was made to administer the ergosterol by injection.

It hardly seems possible that the hypercalcemia obtained in these experiments could have been due to a stimulation of the parathyroids. It is impossible to be assured of the complete removal of all parathyroid tissue by simple thyroparathyroidectomy. However, it would seem most unlikely that an amount so small as to be without effect could upon stimulation by irradiated ergosterol become so active as to produce a marked hypercalcemia in the course of a few days. This is especially true of those dogs in which the abnormally high calcium levels were followed by a hypocalcemia and in some instances by tetany.

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This rules out the possibility of a hyperplasia of the glandular tissue. Furthermore, the additional symptoms accompanying the hypercalcemia produced by overdoses of irradiated ergosterol are not the same as those accompanying the hypercalcemia produced by overdoses of parathyroid extract. In the first place the extent to which the calcium can be raised above normal by feeding ergosterol is greater than that obtained by administration of parathormone. Secondly, Collip, Clark, and Scott (16) have reported that a considerable increase in blood viscosity accompanies the hypercalcemia of parathormone overdosage, and at times they found it almost impossible to obtain a sample of blood. Some samples yielded very little serum even after centrifugation for 1 hour. Hemoglobin determinations throughout our experiments showed no blood concentration, and there was no evidence of increased viscosity. At no time did we experience any trouble in obtaining the blood samples nor in the subsequent separation of the sera. Lastly, according to Collip and associates very definite symptoms such as weakness, vomiting, diarrhea, and possibly death result from overdoses of parathormone if the serum calcium attains a value above 15 mg. per 100 cc. Contrasted to this, the only noticeable symptom accompanying ergosterol hypercalcemia is a loss of appetite which invariably occurred in our experiments. The dogs were always active and playful, and with the exception of refusing to eat, appeared perfectly normal in every respect.

SUMMARY AND CONCLUSIONS.

Preoperative administration of irradiated ergosterol prolongs the life and either prevents or delays the onset of tetany in the thyroparathyroidectomized dog. If tetany occurs, the attacks are usually mild.

The daily administration of 10 mg. of irradiated ergosterol to normal dogs produces a marked hypercalcemia within 2 weeks. The hypercalcemia is invariably accompanied by a loss of appetite. Irradiated ergosterol has no constant effect on the concentration of inorganic phosphorus in the serum.

A marked hypercalcemia can be rapidly produced in parathyroidectomized dogs by the administration of large doses of irradiated ergosterol. Dogs from which the parathyroids have

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been removed can be kept alive indefinitely and apparently in good health by repeated dosage with irradiated ergosterol.

Hemoglobin determinations showed no blood concentration accompanying the hypercalcemia produced by overdoses of irradiated ergosterol. No signs of increased viscosity were observed.

The differences between the hypercalcemias of irradiated ergosterol and parathormone are discussed.

These data show that it is possible to produce a hypercalcemia by feeding large amounts of irradiated ergosterol to parathyroidectomized dogs. In all probability the antirachitic factor does not act by simply stimulating the parathyroid glands to greater activity.

Addendum.—Since the manuscript of this paper was sent to press there has come to the attention of the authors an article by Demole and Christ (Demole, V., and Christ, A., *Arch. Exp. Path. u. Pharmacol.*, **146,** 361 (1929)). The results reported by these investigators agree very closely with the data presented here.

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THE ADAPTATION OF THE BENEDICT-DENIS METHOD TO THE DETERMINATION OF SULFUR IN PLANTS.*

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In an attempt to shorten the procedure for the estimation of sulfur in urine, Benedict (1) used an oxidizing solution containing copper nitrate. Denis (2) modified this solution, and since that time both the original method and this latter modification have been used increasingly in biological work.

Pinchussen and Konarsky (3) used Benedict's original solution for the estimation of sulfur in the organs of animals by adding the solution directly to the finely ground tissue and carrying out the entire procedure in a porcelain centrifuge tube.

Trotman and Bell (4) have used the Denis modification to determine the sulfur in wool, first dissolving the sample in nitric acid. Hoffman and Gortner (5) used the Denis modification to estimate the sulfur in pure organic compounds, after dissolving them in a suitable solvent, and these authors (6) have described the use of the method on plant saps.

Wolf and Osterberg (7) have used the Benedict method after a preliminary treatment of the sample with fuming nitric acid to oxidize most of the organic matter present. This method is complicated by the use of large amounts of fuming nitric acid, and the resulting values for blank determinations are usually high in comparison to the quantity of barium sulfate from the sulfur of most organic materials.

Halverson (8) has further altered this procedure by digestion and disintegration with hot water for 10 hours, and by reducing the amount of fuming nitric acid used in the preliminary treatment.

Even this last simplification does not render the determination

* Contribution No. 389 of the Rhode Island Agricultural Experiment Station.

of sulfur by this method particularly rapid or easy. The digestion with water followed by fuming nitric acid requires about 34 hours, according to the author, necessitating 52 hours for the determination if the worker allows the 18 hour period recommended by Allen and Johnston (9) for the precipitation.

In order to reduce the time for the determination of sulfur in plant materials, and to simplify the method in general, the author has used the Benedict-Denis solution directly on the original sample, without preliminary digestion or disintegration. The results appear to be satisfactory for the crops tested, and they are here presented.

Method.

1 gm. of the finely ground dried plant material is weighed into an 8 cm. porcelain evaporating dish. To this are added 20 cc. of Benedict-Denis solution,[1] and the whole is mixed thoroughly and heated slowly almost to boiling. This temperature (95–100°) is maintained until the liquid is evaporated. It is well to add water from time to time, so that this part of the treatment allows the particles of sample to be infused with the Benedict-Denis solution. This evaporation should require about 1½ hours. After the solution is evaporated to a small volume, heat must be applied carefully in order to avoid spattering. After dryness is reached, the dishes and contents are ignited at dull redness for 10 minutes in an electric muffle furnace, or over a sulfur-free flame.

After cooling, about 50 cc. of 6 N HCl are added to the dish, and the contents heated to dissolve the residue. Unburned particles of carbon are removed by filtration, and the filter is washed well with hot water. 10 per cent barium chloride solution is added to the boiling filtrate (usually 10 to 20 cc. are necessary) and the barium sulfate precipitated in a volume of about 350 cc. and determined in the usual way after standing 18 hours. A blank determination is made for each run, with only the reagents.

EXPERIMENTAL.

The volume of Benedict-Denis solution necessary to hold the sulfur present in 1 gm. of plant material was determined by

[1] The Benedict-Denis solution consists of 25 gm. of Cu $(NO_3)_2$, 25 gm. of NaCl, 10 gm. of NH_4NO_3 dissolved and made to 100 cc. with distilled water. Care must be used in selecting reagents which give low blank values.

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making a study of the sulfur recovered from 1 gm. of cabbage when various amounts of reagent were added. The results are shown in Table I. From these results it is evident that between 10 and 50 cc., the amount of reagent has but little effect on the recovery of sulfur. The quantity to be used was chosen arbitrarily as 20 cc.

In order to determine the accuracy of the method, the results were compared with those secured by the official sodium peroxide method (10). The results are shown in Table II.

The five crops used for this comparison represent five different plant families, and also represent considerable variation in sulfur content. The results by the Benedict-Denis method were obtained by two analysts during a period of more than a year, to eliminate the effects of unrecognized variations from the prescribed technique.

TABLE I.

Sulfur Found in 1 Gm. Sample of Cabbage with Varying Amounts of Benedict-Denis Reagent.

Reagent.	Sulfur.
cc.	*per cent*
10	0.918
15	0.931
25	0.936
50	0.902
Official Na_2O_2 fusion.*	0.924

* *Cf.* (10) p. 44, paragraph 16.

SUMMARY.

It is shown that the Benedict-Denis solution may be used alone as an oxidizing agent for determining sulfur in plants, giving results which are in close agreement with the values obtained by the official sodium peroxide fusion method. The use of any preliminary treatment of the sample is avoided in this procedure, thereby shortening the time necessary for the determination as well as introducing a much simpler technique.

While the results of this work apply strictly only to corn,

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TABLE II.

Comparison of Modified Benedict-Denis Method with Official Sodium Peroxide Method.

Crop.	Sulfur by Benedict-Denis method.	Sulfur by Na_2O_2 method.	Difference.
	per cent	*per cent*	*per cent*
Corn.	0.093		−0.020
	0.098		−0.015
	0.098		−0.015
	0.099		−0.014
	0.111		−0.002
	0.112		−0.001
Average.....	0.102	0.113	−0.011
Buckwheat.	0.082		−0.011
	0.092		−0.001
	0.099		+0.006
	0.102		+0.009
Average.....	0.094	0.093	+0.001
Cabbage.	0.886		−0.038
	0.896		−0.028
	0.924		−0.000
	0.931		+0.007
	0.951		+0.035
	0.973		+0.049
Average.....	0.928	0.924	+0.004
Spinach.	0.408		−0.004
	0.431		+0.019
	0.445		+0.033
	0.447		+0.035
	0.460		+0.048
Average.....	0.438	0.412	+0.026
Onions.	0.613		−0.011
	0.610		−0.014
Average.....	0.612	0.624	−0.013

The probable error of duplicate determinations as calculated by the deviation from the mean = 2.70 per cent.

buckwheat, cabbage, spinach, and onions, it is felt that these crops, representing as they do five different plant families and a

wide range of sulfur content, are representative, and that the methods here used may be applied to any crop with the expectation of reasonably accurate results.

The author wishes to express his appreciation for advice in this work given by Mr. John B. Smith, and for analytical assistance rendered by Mr. Frank S. Schlenker.

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THE RELATION OF THE LIPIDS TO PHYSIOLOGICAL ACTIVITY.

I. THE CHANGES IN THE LIPID CONTENT OF THE CORPUS LUTEUM OF THE SOW.*

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(Received for publication, January 17, 1930.)

The earliest work having to do with the physiology of the fatty substances other than fat was done on the brain by men who were interested in a possible relationship between chemical composition and mental activity (1). The complex nature of the lipid material of the brain prevented much progress in the physiology but stimulated interest in the chemistry of the brain lipids, and much time and effort for many years was devoted to the attempt to separate and identify the lipids of the brain and other tissues. Owing to the difficulty of separation of the lipids from each other and from other substances a large number of compounds were listed, most of which had no real existence, with the result that the chemistry of the lipids was made to appear discouragingly difficult. Recent workers, especially MacLean and Levene have however succeeded in eliminating many of these hypothetical substances and in reducing the chemistry of the group to a relatively simple basis so that there has been recently a renewed interest in their significance in the living organism.

Probably because of their ready oxidation, the possible rôle of the unsaturated phospholipids in cellular oxidations attracted attention first of Koch (2), then Fränkel (3), and more recently Thunberg (4), Warburg (5), Meyerhof (6), and Hopkins (7). The net results of the work of these investigators, while not definitely proving the participation of the lipids in oxidative processes in the living cell, showed that it was very probable.

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* The studies reported in this and the following paper were undertaken in the hope that the data obtained might be of interest in connection with two independent lines of research already under way in the laboratories of the different members of the present group. Since the results obtained would appeal to two widely different groups of investigators it seemed desirable to present the results obtained in two papers and to discuss the findings separately in terms of the two problems.

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The similarity in composition between the phospholipids and the fats led to the belief that the phospholipids were stages in the metabolism of fat. This conception was especially attractive because the miscibility of lecithin with water seemed to offer a means of transport of the insoluble fats. Loew who was the first to state this hypothesis, believed that the phospholipid was a machine for burning the fatty acids. Following him Leathes (8) and a series of later workers succeeded in connecting the phospholipids with some, at least, of the changes undergone by the fat molecule in metabolism. In addition to the work of Leathes and his collaborators may be mentioned the findings of Bang (9) and of Bloor (10) concerning the increases in blood phospholipid during fat absorption, the results of Meigs, Blatherwick, and Cary (11) which indicate that milk fat is formed from the phospholipid of the blood, and the recent work of Sinclair (12) which shows that the fatty acids of the phospholipids of the intestinal mucosa and the liver are promptly changed by the fatty acids of absorbed fat and therefore probably have an important part in fat absorption. The participation of cholesterol in fat absorption, as shown by the increased amount present in the blood during the absorptive period, has been demonstrated by a number of investigators (13).

The conception of the lipids as essential constituents of protoplasm which activated the earliest group of workers in this field has been revived by Mayer and his associates, especially Schaeffer and Terroine. The contributions of this group of workers may be summed up as follows: (*a*) Cholesterol and lipid phosphorus (by inference phospholipids) constitute together an integral and constant component (élément constant) of tissues, the amount of which is not essentially altered by extreme changes in the nutritional status of the animal. These substances are therefore essential protoplasmic constituents—parts of the machine—and not merely fuel. The percentage amounts of these constituents—their "concentration"—is also considered to be characteristic of the tissue. Neutral fat on the other hand is the variable element, not an essential constituent of tissues, and dependent upon the nutritional status of the animal. (*b*) The ratio cholesterol-lipid phosphorus, and in most tissues (those which do not contain much fat), the ratio cholesterol-total fatty acids is constant for and characteristic of the tissue.

Mayer, Rathery, and Schaeffer (14) have shown that the granules and filaments in cytoplasm called mitochondria by the histologists, are lipid in nature, and contain especially unsaturated phospholipid. Thus the considerable information accumulated with regard to the mitochondria has been made applicable more specifically to the lipids. Mayer's conception was that these cellular elements were concerned especially with oxidation. Terroine and Belin (15) considered that the nature of the phospholipid fatty acids was also constant and characteristic, a finding which however has recently been questioned by Sinclair (12).

The conception of a constant and characteristic content of lipids and especially of phospholipids for each tissue, is supported by the work of Bloor (16) who made analyses of various beef tissues of which enough

material was used that the constituents were separated, weighed, and analyzed by standard gross methods, and so avoided the necessity of making assumptions regarding the nature of the compounds. In general it was found that the phospholipid content of a tissue was characteristic of the tissue but that a considerable allowance (30 per cent) must be made for individual variations.

A balanced relationship between cholesterol and phospholipid and less definitely between cholesterol and total fatty acids in blood has been noted by several workers (17–19) and implied by the work of Hueck and Wacker (20) who found that a change in one of the lipid constituents of blood (fat, cholesterol, lecithin) resulted in corresponding changes in the others. Further evidence of a probable balance between these two substances is the fact that cholesterol and lecithin (phospholipid) have been found to be antagonistic in some types of physiological reaction. Thus the hemolysis of red blood corpuscles by lysolecithin is prevented by cholesterol (21). Mayer and Schaeffer (22) found that the red corpuscles of various animals were the more easily hemolyzed as the "coefficient lipocytique," cholesterol-lipid phosphorus, was greater, also that the imbibition of water by tissues varied with this ratio.

Thus not only the concentration of these substances but the balance between them appears to be of physiological significance. What the significance of the balance is can only be guessed, but the phospholipid seems to be the positive factor concerned with these activities and with the general activity of the cell, and cholesterol, therefore, may be considered to be the negative or inhibiting factor. The final degree of activity of a cell would be determined by (*a*) the phospholipid content, (*b*) the phospholipid-cholesterol ratio. The phospholipid content would be a measure of the potential activity; the ratio would indicate the actual or resultant activity.

If we grant that phospholipids and cholesterol are important and integral constituents of protoplasm and concerned with its activities, the next point to be considered is the nature of their relation. Some examples of specific functions of the phospholipids; oxidation, reduction, participation in changes in fats, etc., are mentioned above. Very little is known with certainty about the physiology of cholesterol in spite of the great amount of work which has been done on it. In normal tissues it is present mainly in the free form, while the cholesterol esters are found in notable amounts only in blood plasma and in degenerating tissues such as sclerotic arteries. As noted above, it appears to occur in

definite relationship to phospholipids in tissues, to act antagonistically to the phospholipids in some reactions and to be closely connected to them and to the fats in fat metabolism. Cholesterol prevents hemolysis of red blood corpuscles by free unsaturated fatty acids, possibly by forming combinations with them and it is interesting that the fatty acids in the cholesterol esters of plasma (beef) are highly unsaturated (23). The suggestion of Hanes (24) that cholesterol combines with the fatty acids set free by decomposing lecithin offers a possible explanation of the accumulation of cholesterol esters in degenerating tissues and in blood plasma.

Since we may concede the essentially lipid nature of mitochondria the following conclusions of Cowdry (25) regarding mitochondria may be included here as evidence connecting the phospholipids with general physiological activity.

"In the first place the association of abundant mitochondria [is] with intense protoplasmic activity. In cytomorphosis for example, they are especially numerous in the active stages of the life of the cell and they diminish with senility in both plants and animals. There is a sharp increase in mitochondria with regenerative activity and in compensatory hypertrophy and in many other conditions. There is a distinct reciprocal relationship between the amount of mitochondria and the amount of fat. Where there are few mitochondria, there is much fat and *vice versa*. It seems probable that normal variations in the amount of mitochondria are in some way dependent on variations in the respiration of the cells containing them."

Mayer and Schaeffer, while they emphasize the constancy in content and relationships of the lipids in tissues, give convincing experiments (26) to show that the "normal" values may be changed as the result of changes in environmental conditions, from which the inference may be drawn that the observed constancy is the result of relatively constant environmental conditions. Thus they found that the amounts of lipid phosphorus in the livers and lungs of rabbits were first reduced, then greatly increased as the result of changes in metabolism produced by chilling and overheating. In dogs similar changes were produced, but in the liver only. They quote Richet's data which indicate a correlation between the lipid phosphorus of livers of animals and their size, hence their body surface and total metabolism. Expressed in mg. of lipid phosphorus per kilo of animal Richet's

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values are as follows: beef 0.24, man 0.34, dog 0.41, rabbit 0.61, guinea pig 0.62, mouse 0.69. The variations in phospholipid therefore run parallel with intensity of metabolism. Mayer and Schaeffer (22) and Weill (27) have also presented data to show that the lipid level in parenchymatous tissues is much lower and more irregular in cold blooded animals than in warm blooded animals and lower in hibernants when dormant than when awake.

As the result of analyses of livers of animals under normal and various abnormal conditions Theis (28) found that the normal liver had a higher phospholipid and lower fat content than abnormal liver.

Studies on the lipid content of the more important tissues of a single animal led Bloor to the conclusion that the phospholipid content of a tissue was an expression or a measure of the extent and variety of the physiological activities of that tissue. Thus the liver had a higher phospholipid percentage than the kidney because it had a greater variety of physiological functions; the heart muscle had a higher content of phospholipid than the muscle of the thigh because it was more continuously and strongly active.

The term "physiological activities" as used here is thus meant to include all the processes of the living cell. Of these, oxidation is the basic one, upon which the others depend to the extent at least of their energy requirement, and as noted above oxidation is the function most emphasized in discussions of the mitochondria and the phospholipids. But it should be borne in mind that it is only one form of activity and perhaps not the most important except in muscle where energy transformations are quantitatively the most significant. Yet the muscles (except the heart) have the lowest phospholipid and cholesterol content of all the tissues. In other tissues such as the brain, liver, and kidney having a much higher lipid content, energy transformations are relatively less important and other activities than oxidation must obviously bear greater signficance in the consideration of the total physiological activity of most cells.

In the desire to examine more closely the relation of activity to lipid content of tissues, a tissue was sought which passed through a well defined cycle of changes from inactivity through a period of activity to inactivity again. The corpus luteum of the sow

appeared to be a suitable tissue, and it had the advantage that a complete histological study of its cycle of changes has been made by one of us, both in the non-pregnant and in the pregnant animal (29) as a result of which it is possible to date specimens accurately, in terms of the cycle or of the developing fetus. Moreover, because of the fact that there are several corpora lutea in the same animal at a time, sufficient material for a complete microanalytic study of the lipids can ordinarily be obtained from a single animal.

Regarding the corpus luteum, Fenger (30) found that it contained a relatively high content of phospholipid—about 15 times as much as muscle and about the same as that of other endocrine organs except the thyroid. Chauffard, La Roche, and Grigaut (31) found that the cholesterol content increased continuously during the metabolic cycle of this organ and they suggested that it was one of the places where cholesterol was formed or at least stored. A study of the lipids of human corpus luteum was made by Hermstein (32) and although the amount of available material was small and the difficulty of accurate dating great, his results agreed with those reported below, in that the highest content of phospholipids was found at the time of full activity.

EXPERIMENTAL.

The uteri and ovaries from freshly slaughtered sows were selected at once after removal from the animals and taken to the laboratory where samples of corpus luteum and endometrium were taken (*a*) for histological dating (*b*) for analysis of lipid content and distribution. The details of the histological study and method of dating were reported in a previous paper by one of us (29). It may be stated that the limits of accuracy of the dating on a 21 day cycle is about 1 day except toward the end of the interestrual period, when it may be 2 days. For chemical examination the contents of the corpora lutea, mainly from the same animal, but occasionally from more than one, were shelled out of the capsules, weighed to mg., transferred to a mortar containing about 3 times their volume of clean sand and the whole ground to a fine mud. The material was transferred quantitatively to 200 cc. flasks for extraction, the last traces of tissue and sand being removed from the mortar with the help of 1 or 2 cc. portions of water and rubbing with the pestle. About 150 cc. of 95 per cent

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alcohol were added to the flasks and the mixture boiled for 5 to 10 minutes after which the hot liquid was poured through a filter into 200 cc. graduated flasks. The extracted material, after the alcohol had been drained off, was reextracted several times by boiling out with small amounts of ether, enough ether being used to bring the volume of liquid in the volumetric flasks up to 200 cc. when cooled. From this mixed extract aliquot samples were taken for the determination of phospholipid, total fatty acids, and free and ester cholesterol. Methods for phospholipid (33)

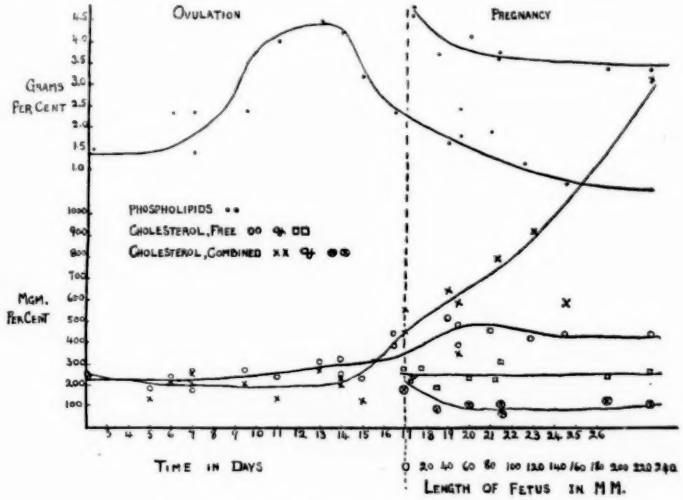

Fig. 1. Lipids of corpus luteum.

and total fatty acids (34) have already been described in this *Journal*. The method for free and bound cholesterol used is new and depends on the oxidimetric measurement of the cholesterol-digitonin precipitate. A preliminary report of the method has already appeared (35) and a full description will be published soon. Comparative determinations by colorimetry were attempted but given up because of the presence of some substance giving an atypical color.

From the values so obtained the ratios phospholipid-free

cholesterol, phospholipid-ester cholesterol, and phospholipid-total cholesterol were calculated. The value for neutral fat was obtained by subtracting from the value for total fatty acids, the

TABLE I.

Corpora Lutea. Non-Pregnant Animals.

Results are expressed in per cent.

Day after estrus.	Sample No.	Free cholesterol.	Cholesterol esters.	Total cholesterol.	Lecithin.	Lecithin / Total cholesterol	Lecithin / Free cholesterol	Lecithin / Ester cholesterol	Fat.	Notes.
2	30–33 (composite).	0.227	0.240	0.467	1.4	3	6.1	5.8	1.16	Normal.
5	27-A	0.194	0.132	0.326	1.43	4.3	7.3	10.8	2.00	Hemorrhagic.
6	35	0 248	0.242	0.490	2.30	4.4	8.3	9.5	2.23	Cystic.
7	X37	0.264	0.200	0.464	2.30	4.9	9.0	11.5	1.76	
7	3	0.173	0.262	0.435	1.42	3.26	8.2	6.0		
9	5					4.6	10.8	7.6		Part lost in extraction.
9–10	15	0.258	0.200	0.458	2.3	5.8	9.0	11.5	0.48	
11	26	0.242	0.117	0.359	4.0	11.5	16.5	34	1.55	
13	4	0.311	0.285	0.596	4.45	7.5	14.3	16.0	2.3	
14	X36	0.314	0.238	0.552	4.2	7.6	13.3	17.6	2.24	
14	25	0.249	0.209	0.458	4.00	8.3	16.0	18.6	2.18	
15	16	0.223	0.105	0.328	3.18	9.7	14.2	30.2	1.8	
16–17	22	0.437	0.472	0.909	2.45	0.6	5.6	5.1	2.19	
16–17	23	0.389	0.545	0.934	2.3	2.4	5.9	4.2	3.27	
19	24	0.52	0.64	1.16	1.66	1.4	3.2	2.6	7.6	
19–20	X29	0.472	0.578	1.04	1.75	1.7	3.5	3.0	3.73	
19–20	28	0.389	0.324	0.713	2.4	3.4	6.2	8.6	3.52	
21	32–33	0.45	0.77	1.22	1.8	1.4	4.0	2.2	3.86	Corpus albicans.
21–24	17	0.41	0.91	1.34	1.18	0.88	2.6	1.2	1.7	" "
24–25	27B	0.43	0.59	1.02	0.71	0.69	1.6	1.2	0.1	" "
25–35	7	0.438	1.75	2.19	1.0	0.45	2.3	0.6		" "

amount of fatty acid in combination in the phospholipid (assumed to be two-thirds of the weight), plus the amount of fatty acid in combination with cholesterol as cholesterol esters $\left(\frac{282}{386}\right.$ or ap-

proximately three-fourths of the weight of the ester cholesterol). This value of residual fatty acids may be multiplied by 1.05 to convert it to glyceride but probably nothing is to be gained by the conversion since the errors of all the determinations are carried by the residual fatty acid. The results are given in Tables I and II and shown graphically in Fig. 1.

TABLE II.

Corpora Lutea during Pregnancy.

Results are expressed in per cent.

Approximate time after estrus.	Length of fetus.	Sample No.	Free cholesterol.	Cholesterol esters.	Total cholesterol.	Lecithin.	Lecithin / Total cholesterol	Lecithin / Free cholesterol	Lecithin / Ester cholesterol	Fat.
day	*mm.*									
7–8	0	11	0.277	0.171	0.448	2.54	5.6	9.0	15.0	3.4
20	3	38	0.213	0.220	0.433	4.6	10.6	14.1	20.9	1.25
17–18		10	0.289	0.056	0.345	4.83	13.6	16.7	86	0.00
	32	9	0.189	0.094	0.283	3.10	10.4	15.7	32	1.38
	60	18	0.236	0.103	0.339	4.2	12.7	17.5	40	2.33
	85	19	0.222	0.104	0.326	3.53	10.8	16.0	34	2.13
	90	1	0.301	0.070	0.371	3.73	10.6	13.1	56	0.93
	190	2	0.240	0.132	0.372	3.3	8.9	13.8	25.4	1.43
	230	8	0.246	0.102	0.348	3.33	9.5	13.8	33.0	2.0
	150 (dead fetus).	12	0.258	0.014	0.272	3.51	12.9	13.6	206	1.77
	160 " "	20	0.298	0.00	0.298	3.62	12.1	12.1	∞	2.48

DISCUSSION.

The changes in lipid content of corpus luteum in this animal may best be followed if they are correlated with the changes in histological structure described by Corner (29). These are briefly as follows: After rupture of the graafian follicle and escape of the egg, the walls of the follicle collapse usually around a central blood clot. The layer of cells surrounding this central cavity then begins to enlarge, apparently by the taking up of material which contains considerable lipid. Within a week the corpora lutea have increased from a diameter of about 4 to 6 mm. to a diameter of 8 to 9 mm. If pregnancy ensues there is a further growth to

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10 to 11 mm. By the 7th day the corpora lutea are usually solid and their cells have become fully differentiated. On cutting the capsule the contents bulge out, are of a yellowish pink color, and in consistency are not unlike pig liver. It is a fact of the greatest importance that the changes which lead up to the formation of the corpus luteum take place with equal completeness whether impregnation has taken place or not. It is not possible to distinguish the corpus luteum of pregnancy from the corpus luteum of unfertilized ovulation during the first 2 weeks after the discharge of the ova. Beginning on the 14th to 15th day a sudden change takes place in the corpus luteum of the unfertilized animals. Within 2 or 3 days the diameter has decreased from 9 mm. to 6 mm.; its color has changed from the pink of active capillary circulation to the whitish tone of scar-tissue and its texture has become much tougher and firmer. Microscopic examination shows that a very rapid degeneration has taken place with complete breakdown of the cells (granulosa) which make up the bulk of the organ and collapse of the capillaries. By the time of the new ovulation (about 21 days) the corpora lutea have diminished in diameter to 6 mm., by the mid-estrual period (30 days) to 4 mm., and by the next ovulation to 2 mm., after which they disappear slowly. The changes may be summed up as follows: 2nd to 8th days rapid growth to full size with beginning function, 8th to 14th days slow or no growth with full functioning; then in the non-pregnant animal, 14th to 21st days retrogression and reabsorption, at first very rapid. In the pregnant animal the corpus luteum remains large and continues its function for a considerable period, perhaps throughout the whole period of gestation, although the diminution in the lipid phosphorus content as observed by Corner (36) may mean diminution of function.

Changes in the Lipid Content.

Phospholipid (Lecithin and Cephalin).—The changes in phospholipid content follow quite closely the gross and histological changes and the functional changes noted above. In the early days the percentage increase is small, then from the 5th to the 10th day the increase is great, while from the 10th to the 14th day, during the period of active function of the gland, there is very little

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percentage change. From the 14th to the 17th day in the non-pregnant animal the decrease in phospholipid percentage is very rapid, running parallel to the retrogression as observed histologically. After that the decrease is slower. If an animal becomes pregnant the phospholipid content of the corpus luteum does not fall on the 14th day. There is apparently an increase up to the time of implantation (17th to 18th day) after which time the phospholipid percentage falls somewhat and then continues at a level slightly below the maximum during as much of the period of pregnancy as our specimens cover. The functional activity and phospholipid content of the gland are both greatest from the 8th to the 14th day in the non-pregnant animal and from the 8th day throughout the gestation in the pregnant animal. The correlation between functional activity and phospholipid percentage is thus seen to be close. The statement of Corner (29) "that by the 7th day the corpus luteum is solid and the cells fully differentiated" apparently does not quite fit in with the facts found about phospholipid, which does not reach its highest values until on or after the 8th day. The discrepancy may however be explained as due to the fact that the cells, although of full size, do not at once reach their maximum content of phospholipid. The percentage of phospholipid in the active gland is about the same as in the liver, perhaps a little higher, which indicates that the corpus luteum may be one of the potentially most active glands of the organism. This high content of phospholipid has been found by Fenger (30) to be characteristic of the endocrine glands with the exception of the thyroid.

Cholesterol (Free Cholesterol).—The percentage of free cholesterol in corpus luteum rises slowly up to the 18th day, then in the non-pregnant animals remains at a fairly constant level, while in the pregnant animals it falls somewhat, and continues throughout pregnancy at about the level found at the 10th to the 14th day. The rise during the development and active life of the gland is much less marked than that of the phospholipid, the maximum difference between high (0.45 per cent) and low (0.27 per cent) values being about 66 per cent of the low value while for phospholipid the difference is about 300 per cent.

Bound or Ester Cholesterol.—In the non-pregnant animals the percentage of ester cholesterol in the corpus luteum remains

practically the same up to the 14th day, then rises rapidly with the retrogression of the gland. In the pregnant animals the percentage of bound cholesterol is at first the same as in the non-pregnant corpus luteum, then at the end of the 3rd week it falls to a low level and remains so low throughout pregnancy that in some cases it is a question whether any at all is present. In the two samples where there were dead fetuses which were being absorbed (Samples 12 and 20) the ester cholesterol was practically absent.

Total Cholesterol.—As was found by Chauffard, La Roche, and Grigaut (31), total cholesterol increases in percentage through the non-pregnant cycle of the corpus luteum. In the period up to and including the time of maximum size and activity of the corpus luteum the increase is slow and due to free cholesterol. During the degeneration the percentage increase is rapid, probably because the cholesterol ester is less readily absorbed and is left behind while the remaining material of the gland is being absorbed (see below).

The notable facts in connection with the cholesterol in this gland are (*a*) the constancy of the free cholesterol percentage while the gland remains active, (*b*) the abrupt rise in ester-bound cholesterol during retrogression. These findings are taken to mean that the free cholesterol percentage is relatively constant in the growing and active gland, and that free cholesterol is supplied by the blood as needed for growth. During retrogression the free cholesterol is either removed or combined with fatty acids which may originate in the lecithin. The failure of the free cholesterol percentage to keep pace with the rapidly rising cholesterol-ester percentage during the period of degeneration may indicate either of these possibilities. The possibility of a deposition of esters from the blood such as Schönheimer (37) finds in arteriosclerosis seems unlikely from a consideration of the change in size of the corpus luteum during about the week's time after retrogression begins. According to Corner's figures the change in diameter is from 9 mm. down to 6 mm. corresponding to a change in volume of from 382 c.mm. to 113 c.mm., or to about 1:3.37. The increase in ester cholesterol percentage is from about 0.2 to 0.7 or about 3.5 times. This would indicate that no deposition from the blood need be assumed, and that the cholesterol and cholesterol esters

found there were residual. The ester cholesterol is thus seen to be low in the corpus luteum during growth, pregestational activity, and in pregnancy (and in the endometrium during its whole life). Its percentage is high in the degenerating corpus luteum apparently because it is left behind during the process of reabsorption. These findings are in agreement with the statements in the literature to the effect that cholesterol esters are present in normal tissue only in small proportions except in the adrenals and the blood plasma. Their presence in considerable amounts in tissues appears to be associated with retrogression in which case they may be either residual or the result of deposition from the blood. Low cholesterol-ester percentage appears to be a sign of activity, high cholesterol-ester a sign of inactivity or retrogression.

Neutral Fat.—Neutral fat is a derived value obtained by subtracting the fatty acids combined in phospholipids and cholesterol esters from the total fatty acids. The value so obtained although it includes small amounts of other fatty acid compounds, is mainly fat. In the non-pregnant corpora lutea as shown by Tables I and II, the percentage of neutral fat is variable but low up till the time of retrogression. Then it increases markedly up to about the 21st day, after which it falls off sharply. The increase probably means that during early degeneration the reabsorption of fat is less rapid than that of some of the other constituents of the degenerating tissue so that the percentage increases. Later, absorption takes place, as shown by the drop in values of fat after the 21st day, while the percentage of cholesterol esters, probably because of less ready absorption, continues to increase. During pregnancy the fat content of the corpus luteum is variable but relatively low.

The corpus luteum has not proved altogether ideal for the study of possible relations between function and lipid content, for the reason that growth and assumption of function and still more the cessation of function and retrogression take place so rapidly. Also the retrogression is really a degeneration followed by ultimate disappearance. The rapidity of the changes has undoubtedly resulted in overlapping of stages in the number of animals which it was necessary to use, and this would explain the divergence of values for given days in the cycle. Nevertheless the results obtained support to a satisfactory degree the hypothesis that the

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phospholipid content of a tissue is a function of its activity. As the corpus luteum grows and becomes active, the percentage content of phospholipid increases until at the time of functioning the percentage is at its highest. This high content persists if pregnancy ensues with consequent continued functioning. If pregnancy does not ensue and functioning ceases, the fall in phospholipid is rapid, until in the gland remnant the content is less than one-fourth of what it was at full activity.

The only other lipid that compared with phospholipid in rapidity and extent of change was the cholesterol ester. But the increase in percentage of this substance occurred only during the degeneration and probably largely because it was left behind in the absorption of the gland material, although possibly also because there was new formation from the free cholesterol and the fatty acids from the disappearing phospholipid. During pregnancy the cholesterol ester content of the corpus luteum was much below what it was in the non-pregnant condition while the free cholesterol was higher. The relation of cholesterol ester to functional activity in this organ was thus negative; its percentage decreased with the activity of the gland and increased with its retrogression. With the decrease of cholesterol esters during pregnancy the free cholesterol increased, indicating that the active gland may keep the cholesterol stripped of fatty acids. The same increase in phospholipid and decrease in cholesterol ester content with activity was noted but to a lesser degree in the endometrium (see following paper). In this organ cholesterol esters were almost lacking during the whole cycle probably because the proportion of degenerating cells in the endometrium of the pig is never great.

The free cholesterol percentage in corpus luteum was about 50 per cent higher during the period of growth, assumption of function, and throughout pregnancy than it was at the beginning, so that its changes do not compare in extent with those of the phospholipid, and its relation to function is therefore not so definitely shown. It may be noted, however, that the percentage content is relatively high compared with that of the liver and kidney.

Before the simple relation of activity to phospholipid and possibly cholesterol content can be accepted certain other factors must be taken into account. These are the relation of the lipids

to growth of the organ and to the hormone formation. Regarding the first of these, the period of growth, according to histological evidence, is usually complete by the 7th day while the increase in percentage of phospholipid continued until about the 13th day, being then nearly double what it was at the 7th day. If pregnancy ensued the high values persisted. The relation of the phospholipid to the functioning of the gland rather than to growth thus seems definite.

With regard to the relation of the phospholipid to the hormone nothing can be said since the nature of the corpus luteum hormone is not known.

SUMMARY.

The percentage content of phospholipid of the corpus luteum of the sow has been found to vary markedly with the activity of the gland, being 2 to 3 times as high during the period of activity (previous to estrus and during pregnancy) as at the time of formation or after retrogression. Free cholesterol increases also up to and during the period of active functioning but to a much smaller extent.

Cholesterol esters were found to vary inversely with the activity of the gland, a high content being characteristic of the degenerated organ.

These results support the hypothesis that the phospholipid content of this tissue is a function of its physiological activity. The relation of free cholesterol to activity is less clear, but appears to be similar to phospholipid. Cholesterol esters on the contrary seem to be related to inactivity or retrogression.

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THE VARIATIONS IN THE LIPIDS OF THE UTERINE MUCOSA IN THE PIG.*

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The structural changes in the uterine mucosa associated with the phenomena of estrus, menstruation, implantation of the embryo, pregnancy, and the puerperium have, for a long time, been of marked interest to the histologist. The staining reactions of the endometrium with Nile blue, Sudan III, Scharlach R, and osmic acid have indicated the possibility of a connection between the lipid content and the state of activity of the tissue. Most of the information we have on the subject is, however, based upon these staining reactions only, largely because of the technical difficulties involved in the study of such tissue by the chemical methods which have been available.

The work of many investigators, beginning with Ancel and Bouin (1) has indicated that the pregestational prolification of the glandular tissue of the endometrium may be dependent upon the action of the hormone of the corpus luteum. Recently Corner and Allen (2) have prepared an active extract of corpus luteum and demonstrated a typical glandular proliferation of the endometrium following its injection into rabbits. This suggests that the cyclic changes in the chemical composition of the endometrium may be expected to run parallel to the various stages of growth and degeneration of the corpus luteum. It has seemed to us, therefore, that parallel chemical studies of the lipids of the

* It is our purpose here to consider the data presented primarily in relationship to the specific physiology of the sexual cycle. For the discussion of the data on endometrium in its relationship to the general metabolism of lipids the reader is referred to the previous paper.

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endometrium and the corpus luteum in the same animals should yield some information of value in the interpretation of the biochemical nature of the phenomena associated with estrus, menstruation, and the nutrition of the fetus.

The whole mechanism for the nutrition of the embryo from the stage of migration of the fertilized ovum to the uterus to the time of birth must function through the uterine mucosa. Moreover, in most animals this tissue undergoes, at more or less regular intervals, a cycle which involves growth and elaboration of special glandular structures, followed by retrogression when the ova are not fertilized. Certain stages of its cycle, *i.e.* estrus and menstruation, are of special interest to the biochemist because they are associated with a condition of congestion or hemorrhage, which in turn must be associated with or dependent upon recurrent changes in the chemical composition of tissue.

The phenomenon of menstruation in the primates, since it is the most spectacular example of this cyclic recurrence of congestion and hemorrhage, has been most frequently studied. The apparent increase in the fragility of the erythrocytes of menstrual blood has recently been ascribed by Hermstein (3) to the increase in the lecithin-cholesterol ratio which he has found in menstrual blood collected directly from the human uterus. His average value for the ratio phosphatide-cholesterol is 1.2 for circulating blood and 1.5 for menstrual blood. His average values for the lecithin-cholesterol ratios in circulating blood are exactly the same during the menstrual period as during the intermenstrual period, although the absolute values for lipid during menstruation are higher. But, since he has only two observations per case in twelve cases altogether, and blood cholesterol varies so much from day to day, it is difficult to see how his figures for circulating blood can have any significance. If, however, we assume that the function of cholesterol is primarily to place a check upon the hemolytic properties of lecithin, or those of the unsaturated fatty acids liberated on the destruction of lecithin, his reasoning seems logical.

Klaus (4) has shown a large increase in the choline content of circulating blood and perspiration during menstruation. But he found in the menstrual discharge itself, trimethylamine, instead of choline. He believes that the menstrual destruction of the uterine mucosa is associated with a breakdown of lecithin, and that the trimethylamine results from bacterial decomposition of choline. An otherwise unaccounted for rise in the total non-protein nitrogen content of circulating blood which has been noted by Okey and Erikson (5) during the first stages of menstruation may likewise possibly be taken as indicative of lecithin destruction.

Okey and Boyden (6) found a considerable increase in the lecithin-cholesterol ratio in circulating blood during the early stages of menstrua-

tion. But this increase in the ratio was due, not to an absolute increase in blood lecithin concentration, but rather to a very marked decrease in cholesterol concentration. These findings have very recently been confirmed by Kaufmann and Mühlbock (7). Later work (Okey, Ehrhardt, and Steinmetz, unpublished results) has indicated that the free, rather than the esterified cholesterol is chiefly involved.

It would appear, therefore, logical to postulate that somewhere in the body there may be a tissue in which cholesterol is used up at an unusually rapid rate at the time of retrogression or destruction of the endometrial cells. This may be merely a mechanism for added defense against bacterial invasion during the period of congestion or hemorrhage. But if this need for cholesterol arises as a result of rapid decomposition of phospholipids with consequent liberation of highly unsaturated or toxic fatty acids which must be rendered harmless by combination with cholesterol, it is reasonable to expect an accumulation of cholesterol ester in this tissue. Corpus luteum, because of its high lipid content, and endometrium, because of the possible intimate connection between the variations in its lipid partition and the production of periodic congestion and hemorrhage seemed worth investigating in this connection. While the animal most readily available for the study, *i.e.* the pig, offered the advantage of a plentiful supply of corpus luteum for chemical analysis from a single animal, nevertheless the comparative simplicity of the cyclic histological changes in the endometrium of this species promised a correspondingly less marked variation in the lipid content. However, the possibility of correlating the findings on the two tissues in the same animal has been considered to outweigh this objection, and the data on endometrium are accordingly presented.

EXPERIMENTAL.

The general method used for collecting and sampling the tissue has been described in the previous paper. After the blocks of corpora lutea and uteri had been set aside for histological study, large pieces of the uteri were taken for the chemical study. Each uterine horn was split open and the mucosa removed as cleanly as possible by dissection with scissors. Except in one case,

each sample was taken from a single uterus. Sample 30–33 was a composite taken from four uteri of the same day.

The endometrical tissue for lipid determination was weighed, ground with sand, and extracted with alcohol and then with ether exactly as described for corpus luteum in the preceding paper. Because of the lower lipid content of the endometrium, it was, however, necesary to use large samples. Moreover, it was soon evident that there was a great variation in water content of the tissue at different stages of the sexual cycle. Hence, separate portions of the samples of uterine mucosa were weighed, dried on the water bath, and then in the desiccator over sulfuric acid, reweighed, and the moisture content calculated in the usual way.

Determinations of the different lipid constituents were made on aliquot portions of the alcohol-ether extracts. The method used for phospholipids was the chromate-sulfuric acid oxidation of Bloor (8) and that used for total fatty acids (9) was based upon the same principle. Cholesterol and cholesterol esters were estimated by micro oxidation of the digitonide according to a new method of which a preliminary report has been published (10). The value for neutral fat was obtained by subtracting from the value for total fatty acids, the amounts of fatty acid in combination in the phospholipid (assumed to be two-thirds of its weight) plus the amount of fatty acid in combination with cholesterol as cholesterol esters, $\frac{282}{386}$ or approximately three-fourths of the weight of the bound cholesterol. This value for residual fatty acids, may be multiplied by 1.05 to convert it to glyceride, but this calculation has not been carried out, because the value for residual fatty acid carries the errors of all the determinations, which may very well aggregate more than this correction.

It should, perhaps, be stated that the analytical determination of lipids in the endometrium presented more difficulties than that of the lipids in the corpus luteum, because of the interference of some non-lipid material, possibly glycogen, soluble in hot alcohol and precipitated from the alcohol-ether solutions on standing. This necessitated refiltration of the extracts of the cooling solution. The analytical results are summarized in Table I.

TABLE I.
Lipid Content of the Uterine Mucosa.

Results are expressed in per cent.

TABLE I.

Lipid Content of the Uterine Mucosa.

Results are expressed in per cent.

Approximate date.	Sample No.	Free cholesterol (moist weight).	Cholesterol esters (moist weight).	Total cholesterol (moist weight).	Lecithin (moist weight).	Dry weight.	Total cholesterol (dry weight).	Cholesterol ester (dry weight).	Free cholesterol (dry weight).	Lecithin (dry weight).	Lecithin.			Fat (moist weight).
											$\frac{\text{Lecithin}}{\text{Total cholesterol}}$	$\frac{\text{Lecithin}}{\text{Free cholesterol}}$	$\frac{\text{Lecithin}}{\text{Cholesterol esters}}$	
Day after previous estrus.*														
21	34	0.10	0.00	0.10	0.5	11.8	0.856	0	0.856	4.4	4.9	4.9	∞	0.26
21	13	0.115	0.016	0.13	0.74						5.7	4.7	46	0.35
Day after estrus.														
2	30–33	0.114	0.033	0.147	0.7	14.5	1.01	0.22	0.79	4.9	4.8	6.1	21	0.23
3	14	0.173	0.025	0.198	1.0						5.0	6.4	40	
6	35	0.162	0.0	0.162	0.7	19.0	0.82	0	0.82	4.9	4.3	4.3		0.97
7	37	0.149	0.0	0.198	1.09	15.0	1.32	0	1.32	7.6	5.8	5.8		
10	15	0.139	0.032	0.171	1.00						5.8	7.2	31	0.24
11	26	0.077	0.040	0.117	0.55	11.2	1.04	0.35	0.69	4.95	4.7	7.1	13.7	0.27
14	36	0.131	0.0	0.131	0.76	13.6	0.96	0	0.96	6.05	5.8	5.8		0.37
14	25	0.082	0.027	0.109	0.54	11.5	0.95	0.23	0.72	4.86	4.8	6.6	20	0.34
15	16	0.126	0.039	0.165	0.6	13.4	1.23	0.29	0.94	4.48	3.6	4.8	15	0.29
16–17	22	0.091	0.004	0.095	0.54	13.2	0.78	0.04	0.74	4.32	5.6	5.9	135	0.21
19–20	28	0.079	0.006	0.085	0.4	11.6	0.73	0.03	0.68	3.6	4.7	5.0	66	0.88
19–20	21	0.106	0.015	0.121	0.6	14.8	0.81	0.10	0.71	4.27	4.9	5.6	40	0
19	24	0.101	0.037	0.138	0.57	10.8	1.28	0.35	0.93	5.13	4.1	5.6	15	0.20
Pregnancy.	10	0.172	0.199	0.371	0.8									0

* Time of estrus.

DISCUSSION.

The cyclic changes in the uterine mucosa of the pig have been described by Corner (11). 3 or 4 days after estrus, proliferation of the glandular cells begins and attains its height at the 8th to 10th day; *i.e.*, when the corpus luteum has become well developed. After the 15th day, *i.e.* at the time of the retrogression of the corpus luteum, there is a slow reversion to the resting type of structure. The changes which take place during the first 15 days are the same whether or not pregnancy follows ovulation. There is considerable congestion during the period just preceding the retrogression of the corpus luteum but never hemorrhage.

Since we find the greatest histological elaboration of the endometrium not at the time of ovulation, but rather at the time when the uterus is ready to receive and prepare for the nutrition of the fertilized ovum, we might expect to find the greatest chemical indication of activity in the endometrium at this time (*i.e.* the 8th to the 12th days). It will be seen from Table I that, in so far as the lipids are concerned, this is the case to a limited extent only.

Total cholesterol on the basis of moist weight is highest from the 3rd to the 10th day, which corresponds to the period of most rapid growth. The highest values for *lecithin* are to be found in the figures for the 7th to the 10th days. The diminution in percentage of *lecithin* after the 15th day is, however, not very striking. The values for the ratio *lecithin* to *cholesterol* are greatest at the period which corresponds to the greatest uterine activity.

Cholesterol esters are present throughout the entire cycle in quantities so small as to indicate no accumulation at any time, save perhaps in the case of very early pregnancy. Hence these data are in accordance with the hypothesis expressed in the previous paper; *i.e.*, that the phospholipid content of a tissue is a function of its activity and that only a small amount of cholesterol esters are found in active tissues.

But, from the point of view of a possible explanation of differences in circulating lipid at different stages of the cycle, our findings are somewhat disappointing. It may be that we are dealing with a tissue in which the blood supply is so great that we can expect no accumulation of lipid material. The fact remains,

however, that the percentage of total lipid present in the endometrium of the pig is approximately that which Bloor (12) has found to be characteristic of lung, kidney, and pancreas of beef, rather than that of the specially differentiated, lipid-rich tissue to be expected from the discussions of Hermstein (3) and Watrin (13) of the human endometrium.

There is a possibility which must be considered, however; *i.e.*, that the lipid content of the endometrial tissue in the pig may differ from that in other species of animals. There is no estrual or menstrual bleeding in the pig. The type of placentation is unusually simple. There is no burrowing of the chorionic villi into the endometrium but only a superficial contact between the membranes of the embryonic sac and the uterine lining. At birth of the young the membranes surrounding the fetus shell out without the extensive tearing of uterine tissue characteristic of most other species. Moreover, the amount of glandular proliferation in the endometrium at the time of implantation is less in the pig than in species with more elaborate placentation. Hence it is possible that lipid changes in the glandular tissue of our samples may have been masked by the high proportion of comparatively inert connective tissue. For this reason, we hope to make similar investigations of the lipids of this tissue in other species. But because this will have to be done in separate laboratories and because the work herein reported constitutes an integral part of our original investigation we are presenting the lipid figures for the endometrium of the pig together with those for the corpora lutea of the same animals.

The relative amount of corpus luteum tissue which may be present in the ovaries of a single animal is, on the other hand, greater in the pig than in most other species. But the total mass of corpus luteum tissue which we were able to obtain from any one animal at the stage of greatest development (15th day) averaged approximately 6 gm. and contained 0.4 to 0.6 per cent = 0.024 gm. of total cholesterol and approximately 4 per cent = 0.24 gm. of lecithin. Retrogression of the corpus luteum results in an increase in the percentage of cholesterol but in a decrease in total mass which more than compensates for it (see previous paper). Consequently we are justified in concluding that in the pig there is not at any stage of the sexual cycle an

accumulation of cholesterol sufficient to account for any considerable change in its concentration in the blood stream.

SUMMARY.

The total lipid content of the uterine mucosa of the pig on the basis of dry weight, is approximately the same as that of lung, kidney, and pancreas of beef. The water content of this tissue is variable and high, especially during the stages of congestion.

There is a definite but not large increase in the percentage of lecithin at the time of greatest elaboration of the endometrium. This is, however, accompanied by an increase in free cholesterol. Hence, the lecithin-cholesterol ratios are not as high as those in the corpus luteum at the same time.

The amount of cholesterol ester present in the endometrium is always very low. There is never any indication of accumulation of this substance in the endometrial tissue. There is, moreover, too little accumulation of cholesterol in the corpus luteum of this species to account for any considerable change in the cholesterol concentration of the blood.

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CYSTINURIA.

THE EXCRETION OF A CYSTINE COMPLEX WHICH DECOMPOSES IN THE URINE WITH THE LIBERATION OF FREE CYSTINE.

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In spite of many detailed investigations on cystinuria, its mechanism still awaits elucidation. The following study suggests a new angle from which this baffling problem may be approached. Since a number of good reviews (7, 11, 14, 21, 23, 30, 33) of the subject are easily accessible, we shall omit a historical introduction.

One of us (Biloon) had made a diagnosis of cystinuria in a private patient on identification of a urinary calculus as cystine. A freshly voided specimen of urine, examined on the patient's first visit to the laboratory, was found to be clear, free from albumin and sugar, and strongly alkaline[1] (pH = 8.9). The lead acetate test was strongly positive, which seemed to indicate the presence of large amounts of cystine. The Sullivan reaction (36–38), which is specific for free cystine, was negative, contrary to all expectations. The characteristic red color of this reaction, which usually develops in from 10 to 20 minutes, did not appear and was still absent after 2 hours. The following day it was found that a brilliant red color had developed which continued to increase in intensity for the next 24 hours and then gradually faded.

This peculiar behavior of the Sullivan reaction and its implications were the basis for devising new experiments in an attempt to gain further insight into the obscure nature of cystinuria.

Due to lack of space the history of the case is omitted. It is of interest to note, however, that a dissimilar twin brother of the subject is not a cystinuric.

[1] Patient had been taking large doses of sodium bicarbonate.

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Our plan to conduct a thorough investigation of cystinuria was unfortunately frustrated because the patient could not cooperate with us for more than a few days on account of interference by the family.

EXPERIMENTAL.

1. Miscellaneous Observations.

A number of specimens of urine with a pH of about 6.2 were clear when freshly voided. When the urine was left standing at room temperature, with toluene as a preservative, a precipitate settled out in the course of several hours which continued to increase during the next 24 to 36 hours. This precipitate was almost pure cystine, microscopically (hexagonal platelets) and chemically.

The addition of trichloroacetic acid and of sulfosalicylic acid to freshly voided urine produced no precipitate. A search for substances which could be precipitated isoelectrically in freshly voided urine was negative. A search for insulin-like bodies was also negative (rabbit test). Concerning the presence of other amino acids, it was observed that a number of specimens of urine gave a positive Millon and a positive Sakaguchi (34) reaction.[2] These reactions were not strong enough to indicate the presence of appreciable amounts of either tyrosine or arginine. No free tyrosine was found in a casual specimen which was tested by the new tyrosinase method (Lichtman, S. S., and Sobotka, H., *J. Biol. Chem.*, **85**, 261 (1929)). We are indebted to these authors for carrying out the determination.

2. The Folin-Looney Reaction.

The Folin-Looney (9, 10, 27) test was not applied to cystinuric urine since it has been established that this reaction is not specific for free cystine. Thus dialanyl-cystine, glutathione (20), insulin

[2] We find that the Sakaguchi reaction is positive in a number of normal, protein-free urines decolorized with charcoal. In this connection Hoppe-Seyler's (19) observations on the isolation of pure arginine from cystinuric urine are of interest. The difficulties, which he encountered, may have been due to arginine being present originally in a combined form. According to our experience, arginine, if present in sufficient amounts, may be isolated from urine as the insoluble benzaldehyde-arginine (*cf.* Bergmann and Zervas (3)).

(42), a solution of wool in HCl (31), ergothioneine (39), and other compounds (39) give a positive reaction. Some of these substances, however, develop less color than the equivalent amounts of cystine.

Folin (10) has recently improved the reagent for the cystine determination. It was found that with the new reagent equivalent amounts of cystine and of cystine dimethyl ester dihydrochloride[3] develop exactly the same amount of color. Dialanyl-cystine, however, develops much less color than cystine; 2, 4, 6, and 10 mg. of this substance read equivalent to 0.76, 1.05, 1.14, and 1.34 mg. of cystine respectively.

3. The Cyanide-Nitroprusside Reaction as a Test for Cystinuria.

We believe that the following simple test, used judiciously, will be of value in detecting cystinuria.

Take 3 to 5 cc. of urine, add 2 cc. of a 5 per cent solution of NaCN and allow to stand for 5 to 10 minutes. Then add a few drops of a 5 per cent solution of sodium nitroprusside. Normal urines develop only a faint color, while cystinuric urines give a rather stable magenta color, which is characteristic for SH groups. The differentiation is possible, because Weyl's reaction for creatinine and Legal's for acetone are very faint with NaCN, and develop only on the addition of NaOH or NH_4OH. For reasons not entirely elucidated (18) cyanides reduce S–S groups to S–H groups and also prevent the reoxidation of the S–H groups (29). The test is simple to perform and should be carried out in the routine examination of the urine of large groups of individuals. In cases of renal colic the test, if positive, should be of diagnostic value.

4. Metabolic Observations.

The metabolic observations, although of short duration, contain some points of sufficient interest to be recorded. The patient was on a diet of about the same character throughout the period and weighed his food. 24 hour specimens of urine were collected.

Table I shows that our case does not differ materially from the majority of those described in the literature, the neutral sulfur

[3] It is interesting to note that the sulfur in cystine methyl ester is more labile than in cystine (*cf.* Brand and Sandberg (5)).

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TABLE I.

Metabolic Observations.

Date.	Diet.				Urine.																							
								Creatinine.		Nitrogen.									Inorganic phosphates as P.	Sulfur as S.				In per cent of total sulfur.				
	Carbohydrate.	Protein.	Fat.	Sulfur.	Volume.	Specific gravity.	pH	Preformed.	Total.	Total.	Urea.	Ammonia.	Creatine + creatinine.	Uric acid.	Amino acid.	Undetermined.	Undetermined in per cent of total.		Total S.	Inorganic sulfates.	Ethereal sulfates.	Neutral S.	Inorganic sulfates.	Ethereal sulfates.	Neutral S.	Neutral S calculated as cystine.	Cystine N calculated from neutral S.
1929	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*			*gm.*	*gm*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*
Jan. 8	300	100	80	1.25	1340	1018	6.2	1.51	1.56	11.30	9.20	0.28	0.58	0.09				0.77	0.810	0.407	0.084	0.319	50.2	10.4	39.4	1.20	0.14
" 10	340	125	125	1.38	1680	1018	6.2	1.51	1.57	12.22	9.83	0.33	0.58	0.12				0.94	0.932	0.457	0.076	0.399	49.0	8.2	42.8	1.51	0.18
" 16*	290	120	70	1.48	1900	1018	6.8	**1.86**	**1.92**	11.63	9.12	0.30	0.71	0.13	0.37	1.00	8.6	0.91	0.994	0.475	0.072	0.447	47.8	7.2	45.0	1.69	0.20
" 18	230	115	80	1.25	1540	1020	6.2	1.59	1.68	11.30	8.73	0.31	0.62	0.12	0.36	1.16	10.3	0.82	0.895	0.474	0.073	0.348	53.0	8.1	38.9	1.31	0.15

* 2 hours of exercise (handball).

being about 40 per cent of the total sulfur. The undetermined nitrogen amounts to about 9 to 10 per cent of the total nitrogen. This figure is slightly higher than in the normal and is in agreement[4] with the findings[5] of other workers (2, 43, 44).

The daily creatinine excretion is uniform except on January 16, when there is a marked rise. That this rise is not due to a bad partition of the urine can be seen from an analysis of the figures. It is interesting to note that on this day the patient indulged in 2 hours of exercise (handball). This unusual rise in creatinine excretion following exercise is in agreement with the more detailed and more conclusive observations of Alsberg and Folin (2) upon this point. The apparent lability of the creatinine excretion in patients with an abnormal sulfur metabolism may be physiologically significant and deserves further investigation.

5. The Sullivan Reaction.

Sullivan (36–40) has recently described a new color test for cystine, which has proved, thus far, to be specific. Cystine derivatives such as glutathione and insulin do not give the reaction, although they are positive with the Folin-Looney test.

We find that cystine dimethyl ester dihydrochloride gives a positive Sullivan reaction. Preliminary observations seem to indicate that the ester may develop a color which is not only more intense but also more stable than that produced by equivalent amounts of cystine.

We further find that dialanyl-cystine does not give a positive Sullivan reaction even if left in contact with the reagents at Step II of the reaction (see below) for 24 hours. In this connection it may be pointed out that Sullivan has found that glutathione gives a slight red color after standing in contact with the

[4] We find unusually high figures for undetermined nitrogen in a recent paper by Robson (32). On the assumption that his patient excreted daily 0.80 gm. of creatinine plus uric acid nitrogen (a high estimate), we calculate that the undetermined nitrogen rises in the control period (Table I) from 35 to 57 per cent of the total nitrogen. In Table II are undetermined nitrogen values as high as 60 per cent. On the last day of cystine administration (Table IV) the undetermined nitrogen was 31 per cent and dropped on the following day to 5 per cent. Unfortunately, the author did not comment on these and other unusual findings.

[5] Corrected for amino acid nitrogen.

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reagents for 3 to 4 hours (37). This is another indication of the relative instability of the glutathione molecule (5). The effect of freshly voided urine on dialanyl-cystine was also investigated. Normal freshly voided urine (pH = 5.5) plus dialanyl-cystine incubated at 37° for 15 hours, with toluene as a preservative, gave a negative Sullivan reaction.

The Sullivan reaction may be used for the quantitative determination of cystine, colorimetrically. Checks may be obtained after a certain amount of experience with the method. Pyrex test-tubes,[6] graduated at 25 cc., should be used in the determination. We have modified Sullivan's procedure in some minor details to meet our particular needs.

Procedure.

Step I.—To the standard or unknown, made up to 5 cc., add 2 cc. of a 5 per cent solution of NaCN and allow to stand for exactly 10 minutes.

Step II.—Now add 1 cc. of a 0.5 per cent solution of 1.2 naphthoquinone-4-sodium sulfonate (8), mix, and add immediately 10 cc. of a 10 per cent solution of $Na_2SO_3 \cdot 7\ H_2O$ in 0.5 N NaOH, mix by inverting, and allow to stand 30 minutes to 1 hour, well stoppered and preferably in the dark.

Step III.—Now add 1 cc. of a 2 per cent solution of sodium hydrosulfite ($Na_2S_2O_4$) in 0.5 N NaOH, mix, dilute to the mark with 0.5 N NaOH, invert twice, and read immediately.

In Step II sodium sulfite should follow the reagent as quickly as possible. It was found that better checks were obtained when the sodium sulfite solution was added almost simultaneously to duplicate tubes. All steps in the determination should be carried out in the same manner for both standard and unknown. Readings should be taken immediately, since the color starts to fade in about 20 minutes.

The standard used is a solution of *l*-cystine in 0.1 N HCl, containing 2.5 mg. of cystine per cc. Proportional amounts of color are developed with 0.5 to 1.5 cc. of the standard, containing from 1.25 to 3.75 mg. of cystine. The amount of cystine in the unknown should be within this range, preferably about 2.5 mg. All

[6] We are under the impression that tubes which have been repeatedly used in this determination may give better checks.

the solutions except the NaCN solution should be freshly prepared. The standard should not be kept longer than a week.

Experiments were carried out in order to learn whether cystine added to normal urines could be determined by this method. In most of these experiments 2.5 mg. of cystine were added to 3 cc. of urine. When the Sullivan reaction was carried out with urine plus cystine it was observed that following the addition of the NaCN in Step I a white, flocculent precipitate appeared which usually went into solution in Step II, but sometimes only in Step III. It was further observed that in Step II a certain amount of brown color appeared in addition to the characteristic red color due to cysteine. This brown color varied in intensity with different urines and disappeared, with rare exceptions, in Step III.

It was also noticed that the presence of urine produced a definite slowing of the reaction in Step II. In order to determine whether any of the usual urinary constituents were responsible for this effect, the following substances were tried: creatine, creatinine, urea, disodium phosphate, and uric acid. None of these substances produced any such slowing effect except uric acid and that only when added in larger amounts (2 to 5 mg.), smaller amounts (0.5 to 1.5 mg.) having no effect. The amount of uric acid present in the urine samples, as determined by the Benedict-Franke method, was not sufficient to account for the slowing effect observed. The possible influence of NH_3 as a factor was also eliminated by adsorbing the ammonia with the aid of permutit. Sullivan independently observed that urine interferes with the quantitative cystine determination by his method. The interfering material he could remove by carboraffin ((40) and personal communication).

While cystine standards develop a maximum amount of color, if 10 to 20 minutes are allowed at Step II, cystine plus urine requires about 1 hour at this step. This maximum, however, is equivalent to only 90 to 95 per cent of the cystine which has been added. The red color, which develops in cystine standards, fades several hours after the sodium sulfite has been added (Step II). In the presence of urine, however, the color is quite stable and even after standing for 24 hours 60 to 80 per cent of it is still present. One should consider the possible presence in normal urine of a

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cystine complex, from which cystine is gradually liberated, to account for this behavior.

It has been claimed that small amounts of cystine are present

TABLE II.

Sulfur Partition of Fresh Specimens of Urine.

Specimen.	Age of specimen.	Date.	Volume.	pH	Uric acid.	Sulfur as S.				In per cent of total sulfur.			Neutral S calculated as cystine.
						Total S.	Inorganic sulfates.	Ethereal sulfates.	Neutral S.	Inorganic sulfates.	Ethereal sulfates.	Neutral S.	
		1929	*cc.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*				*mg.*
Urine A.	3 hrs.	June 7	200	6.4	72	148	74	11	63	50	7.4	42.5	236
" B.	3 "	" 21	340	7.8	95	189	80	12	97	42.3	6.4	51.3	364

TABLE III.

*Effect of Age and Acid Hydrolysis on Cystine Determinations.**

	Age of urine specimen.	Mg. of cystine in specimen (in per cent of neutral S).		
		1 hr. at Step II.	5 hrs. at Step II.	24 hrs. at Step II.
Urine A (neutral S = 236 mg. cystine).	3 hrs.	101 (43)	124 (53)	172 (73)
	3 " hydrolyzed with HCl. Not decolorized.	185 (78)		
	8 hrs.	123 (53)	142 (62)	
	24 "	157 (67)	165 (70)	
Urine B (neutral S = 364 mg. cystine).	3 hrs.	213 (59)	281 (77)	374 (103)
	3 " hydrolyzed with HCl. Decolorized with charcoal.	244 (67)	230 (63)	
	7 hrs.	278 (76)		380 (104)
	24 "	334 (92)	329 (90)	

* Urine specimens same as in Table II.

in normal urine (13, 28). Since the completion of our experiments Sullivan (40) has reported that a cystine compound is present in normal urine.

While the recovery of cystine added to urine varies within the above mentioned limits with different specimens of urine, still fair checks can be obtained with the same specimen. The age of the specimen of urine was not found to have any influence upon the determination.

With this knowledge on the behavior of the Sullivan reaction with cystine in normal urine, we felt we were in a position to employ this reaction in an investigation of cystinuric urine. It was considered exceedingly important to carry out, with the aid of the Sullivan reaction, cystine determinations not on 24 hour, but rather on freshly voided specimens of cystinuric urine, also on portions of the same specimens after standing for varying intervals, and after acid hydrolysis. It was further thought advisable to carry out neutral sulfur determinations in order to have some estimate of the maximum amount of cystine which could be present. Two such experiments are described in Tables II and III.

Both samples of urine were morning specimens. Urine B was more alkaline because the patient had taken some sodium bicarbonate the previous day.

In some of the determinations more than 1 hour was allowed at Step II of the Sullivan reaction (see procedure above) which was then completed as usual. This is designated in Table III as 1 hour at Step II, 5 hours at Step II, and 24 hours at Step II.

The values for cystine reported are with few exceptions the averages of duplicate determinations carried out with two different amounts of urine and read against two different standards.

For the acid hydrolysis 40 cc. of urine were boiled for 2 hours with 10 cc. of concentrated HCl. This was then neutralized, made up to 100 cc. with water, and suitable amounts taken for cystine determinations. The hydrolyzed specimen from Urine A was not decolorized with charcoal and, therefore, 2 cc. of hydrosulfite were used in Step III of the Sullivan determination in order to destroy some of the brown color. This was, however, not entirely satisfactory and the colorimetric readings were rather difficult. The hydrolyzed specimen from Urine B was decolorized with liberal amounts of charcoal. It is well known that cystine is adsorbed in appreciable amounts by charcoal (10). Therefore, the amount of cystine present in Urine B, after hydrolysis, was probably much greater than the amount actually determined.

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From Table II it can be seen that the amount of neutral sulfur in Urines A and B was 42 and 51 per cent of the total sulfur respectively. From Table III it can be seen that the amount of cystine determined according to the procedure as outlined above increased with the increasing age of the specimen. Thus after standing for 24 hours at room temperature the amount of cystine found in Urine A was 157 mg. and in Urine B 334 mg.; whereas, after 3 hours there was found only 101 and 213 mg. respectively, an increase in both instances of approximately 55 per cent. The amount of cystine found, after 24 hours, in Urine A was equivalent to 67 per cent of the neutral sulfur and in Urine B to 92 per cent.

It should be further noted that the amount of cystine determined increased with the time allowed for Step II of the Sullivan reaction. Thus, *e.g.*, the 3 hour specimen of Urine B gave 213 mg. after 1 hour at Step II and 374 mg. after 24 hours at Step II, an increase of 75 per cent in the cystine determined. It should be pointed out, however, that with the older specimens this rise produced by longer standing at Step II was very slight or nil. Thus with Urine B 24 hours old, there is practically no change since 90 per cent of the neutral sulfur is already accounted for after 1 hour at Step II.

Following acid hydrolysis larger amounts of cystine are found than by direct determination in the same specimens (Table III). It is evident, therefore, that the cystine content of one and the same sample of cystinuric urine, as determined by the specific Sullivan reaction, is extremely variable, amounting to from 40 to 100 per cent of the neutral sulfur. This variation appears to depend upon a definite time factor since as the urine becomes older its cystine content increases until a maximum is reached.

Two explanations seem possible to account for these findings: First, the presence in the cystinuric urine of a substance which inhibits the Sullivan reaction and the effects of which gradually disappear. It should be pointed out, however, that normal urine does not contain any such gradually disappearing inhibitory substance. Second, the presence of a cystine complex which gradually splits off cystine. The finding of free cystine in freshly voided urine would not invalidate this explanation, because a cystine complex which would decompose so readily in voided urine could also decompose in the urinary tract. Thus even freshly voided

urine could contain free cystine. Although no such cystine complex has been isolated from cystinuric urine, we are inclined to conclude from our experiments and from further available evidence which will now be discussed, that the second explanation is the correct one.

DISCUSSION.

It had been noted, even by the earliest observers, that the precipitation of cystine from cystinuric urine took place very gradually and that the addition of small amounts of acetic acid hastened and increased this precipitation. Up to the present time this phenomenon has been explained as a crystallization of cystine from a supersaturated solution and it has been generally assumed that all of the cystine which crystallizes out is already present as such before crystallization. That smaller amounts of cystine settled out from alkaline urines than from neutral or slightly acid ones was explained by the greater solubility of cystine in an alkaline medium.

A dissenting view, however, can be found in a preliminary communication by Delépine (6) in 1890, which seems to have been ignored by subsequent investigators. He suggested "*that the theory generally held as to the state of combination of cystin in the urine is probably inaccurate*" and offered as the most probable explanation of his experimental findings "that a compound exists in certain urines which under the *influence of a fermentation* yields cystin." Delépine's observations and conclusions are exceedingly interesting in view of our findings and should be reinvestigated in the light of present knowledge of the urinary enzymes.

Following the continued feeding of sodium bicarbonate to a cystinuric patient, Klemperer and Jacoby (22) observed that not only did the cystine crystals disappear from the urine, but also that the cystine, as determined by the Gaskell (12) method, gradually diminished and finally disappeared entirely from the urine. Unfortunately these authors did not carry out any neutral sulfur determinations, nor did they investigate whether the cystine had possibly been excreted in some other form which was not extracted by the Gaskell method.

Smillie (35) confirmed the findings of Klemperer and Jacoby in regard to the disappearance of the cystine crystals from the

urine when large doses of alkali are administered. He found, however, that the neutral sulfur remained unchanged and, therefore, concluded that the cystine excretion remained unaltered. That such a conclusion cannot be drawn without reservations is illustrated by the findings of Looney, Berglund, and Graves (28) which will be discussed later.

Hele (17) observed in two cases of cystinuria that although the amount of cystine, determined by precipitation, was about the same, still the neutral sulfur in one case was much higher than in the other. He states, "This difference would suggest some abnormality of sulphur metabolism other than that involved in the excretion of cystin, provided there is no great error in estimation." Williams and Wolf (43) found that the output of neutral sulfur from which the cystine was subtracted was abnormally high and concluded that this was due either to the increased excretion of a normal constituent of urine or to some abnormal neutral sulfur compound other than cystine.

Mackenzie Wallis and Garrod ((12) p. 110) reported a case in which cystine could not be detected by the Gaskell method, but in which cystine crystals were deposited in the urine after 6 months standing. Numerous similar observations can be gathered from the literature. In summarizing on this point, Garrod (12) states: "Unless we are prepared to admit that precipitation methods may wholly fail even when cystin is abundantly present as such in urine, the inference cannot be avoided that under certain conditions cystinurics excrete a sulphur compound which is not free cystin but a compound or unoxidized derivative thereof."

More recently, Looney, Berglund, and Graves (28) found that the administration of sodium bicarbonate to a cystinuric patient on a standard diet caused some remarkable urinary changes. Although there was a drop in the excretion of the total nitrogen and the total sulfates, still the excretion of the neutral sulfur remained fairly constant during the period of alkali administration, whereas the cystine crystals disappeared from the urine and the colorimetric determination showed decreasing amounts of cystine. The ratio between cystine sulfur and neutral sulfur thus dropped from 100 to 28 per cent. The authors state that the bicarbonate unquestionably alters the metabolism of cystine sulfur, but that the manner in which this change is accomplished is

completely hidden. They surmise, however, that there may be a combination of the cystine which cannot be determined by their method. Although the authors believed, at that time, that the Folin-Looney method indicated free cystine, it was subsequently shown that cystine compounds also give this reaction. Some of these compounds, however, give much less color than that which equivalent amounts of cystine produce (see p. 317). The observation, therefore, of a marked decrease in cystine without a corresponding drop in neutral sulfur could be explained by the presence of a cystine complex. It should also be pointed out that the constancy of the neutral sulfur excretion in these experiments would seem to indicate that alkali administration has no peculiar effect on the cystine sulfur metabolism, but rather on the stability of the cystine complex in the urine. Neubauer (30) summarizes the findings of Looney, Berglund, and Graves as follows: The decrease of cystine excretion following alkali administration is due to a replacement of the cystine by another substance which is not yet known but which belongs to the neutral sulfur group.

Highly suggestive evidence of a cystine complex is also found in the careful metabolic studies (2, 22, 28, 43, 44) on cystinuric patients. On a standard diet such patients excrete daily fairly uniform amounts of neutral sulfur. An increase in the protein (*i.e.* combined cystine) intake is followed by an increase in the neutral sulfur and cystine excretion and a decrease in the protein intake is followed by a decrease in the neutral sulfur and cystine. But if free cystine is fed, the neutral sulfur excretion remains fairly constant and little if any additional cystine appears in the urine, for the cystinuric patient oxidizes free cystine to about the same extent as the normal and excretes it as inorganic sulfate.[7] Investigators have always been at a loss to explain how a cystinuric patient on the one hand can oxidize the free amino acid cystine, when it is fed as such, and on the other hand cannot oxidize the same amino

[7] Loewy and Neuberg (26) reported a case of cystinuria which was able to burn "stone cystine" but not "protein cystine." It was subsequently shown, however, that protein and stone cystine are identical and one is at a loss to understand why their patient could burn the one and not the other. Furthermore, the method used for determining cystine in the control period and in the period of "stone cystine" feeding was different from that used for the period of "protein cystine" feeding. The findings are, therefore, difficult to evaluate.

acid when it results from the feeding of protein. A feasible explanation becomes available, if the cystine is excreted not as such but as a cystine complex which under the conditions usually prevailing in the urine, decomposes with the liberation of free cystine.

In regard to the nature of such a cystine complex it is plausible to assume that it is either a peptide or a simple protein since it would be derived from protein metabolism, either exogenously or endogenously (24). Such a view is supported by the high values for amino acid and undetermined nitrogen in cystinuria and also by the isolation of other amino acids from cystinuric urine. In this connection glutathione or the glutathione-containing complex should be kept in mind as a possibility.

The cystine complex is apparently very unstable, since it undergoes spontaneous decomposition on standing at room temperature. This instability may be a contributing factor in the cystine calculus formation so frequently observed in cystinuria. Also the reagents in the Sullivan test seem to decompose the cystine complex gradually. This is similar to the behavior of glutathione as observed by Sullivan.

If the cystine complex is a peptide or a simple protein, its spontaneous decomposition in urine may be due to the action of the urinary proteases. The presence in urine of a number of proteolytic enzymes with different pH optima is well established (15, 16) but little is known about their nature and their excretion under varying conditions. Thus the effect observed with alkali feeding in cystinuria, which apparently increases the stability of the cystine complex, may be due to an interference either with the action or with the excretion of the urinary enzymes. Such an effect, however, may be only transient owing to an adaptation of the organism. Sylla (41) believes that in some instances alkali administration may bring about a solution of cystine stones with a concomitant increase in cystine excretion.

It had long (2) been inferred from the metabolic studies in cystinuria that the cystine of the protein molecule is absorbed in the form of a higher complex and not as the free amino acid, but one was reluctant to admit the importance of these findings for the general problem of protein absorption (30). Since in the light of the previous discussion the apparently paradoxical behavior

of the cystinuric becomes quite intelligible, the metabolic findings in this disease give added weight to the evidence which is accumulating from a variety of sources that proteins may be absorbed in some complex form to a greater extent than is generally realized.

The metabolic studies indicate that the cystine complex excreted in cystinuria is partly endogenous and partly exogenous in origin. Whether or not exogenous and endogenous protein metabolism are such separate and distinct processes as is generally assumed, and the bearing of cystinuria upon this question, will be discussed subsequently in connection with our studies on the origin of creatine (preliminary communication (4)).

The following possibilities may be considered to explain the excretion of a cystine complex by the cystinuric. (1) The presence in excessive amounts (due either to increased formation or decreased destruction) of a normally occurring compound, part of which escapes into the urine.[8] (2) The formation of an abnormal compound to which the kidney is permeable.[8] (3) An increased renal permeability to a normally occurring compound as *e.g.*, renal diabetes. (4) Any combination of these several factors.

Regarding the diagnosis of cystinuria the presence of free cystine in the urine should not be considered as a *conditio sine qua non*.

The term intermittent cystinuria should be restricted to those cases in which for various reasons the neutral sulfur excretion (due essentially to cystine in some form) rises temporarily to abnormal levels and subsequently returns to normal.

We hope that a better understanding of the nature of cystinuria will result from future investigations along the lines suggested by this study.

SUMMARY.

1. The importance of the cyanide-nitroprusside test for the detection of cystinuria and the diagnostic value of the test in cases of renal colic is pointed out.

[8] The fact that free cystine has been found in the tissues of four cases of cystinuria post mortem (1, 25) would be in harmony with either of the possibilities 1 or 2. However, it should be pointed out that these cases were children who died with complications which may have accounted for the findings.

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2. The behavior of different sulfur compounds in the Folin-Looney reaction is discussed.

3. The Sullivan reaction was used for the quantitative estimation of cystine in cystinuric urine.

4. Evidence for the probable presence of a cystine complex in normal urine is discussed.

5. Evidence is assembled from the literature and, with the aid of the Sullivan reaction, new evidence is presented which indicates that in cystinuria a cystine compound is excreted which decomposes in the urine with the liberation of free cystine.

6. It is pointed out that the excretion of a cystine complex in cystinuria could result from (1) the presence in excessive amounts (due either to increased formation or decreased destruction) of a normally occurring compound, part of which escapes into the urine, from (2) the formation of an abnormal compound to which the kidney is permeable, from (3) an increased renal permeability to a normally occurring compound as *e.g.*, renal diabetes, or from (4) any combination of these several factors.

7. It is pointed out that free cystine in the urine is not essential for the diagnosis of cystinuria. It is pointed out that the term intermittent cystinuria should be restricted to cases with a transient high neutral sulfur excretion.

8. The bearing of the metabolic observations in cystinuria upon the problem of protein absorption is discussed.

9. It is pointed out that the apparent lability of the creatinine excretion in patients with an abnormal sulfur metabolism may be of physiological significance.

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THE CHEMISTRY OF JAFFE'S REACTION FOR CREATININE.

VI. A COMPOUND OF PICRIC ACID WITH TWO MOLECULES OF CREATININE. ITS COMBINATIONS WITH ACID AND ALKALI.*

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(From the Littauer Pneumonia Research Fund, New York University, New York.)

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In Paper IV of this series (1), there was described a compound containing 2 molecules of creatinine, 1 of picric acid, 3 of sodium hydroxide, and 3 of water. This was precipitated by running into alcohol the mixture obtained by the reaction of 1 mol of picric acid with 2 or more mols of creatinine and an excess of sodium hydroxide. The creatinine in this compound did not give Jaffe's reaction until after its solution in dilute acetic acid had been allowed to stand for some time.

In another paper (2) it was shown that when the alkaline reaction mixture contained an excess of picrate over creatinine, precipitation with alcohol yielded a red substance which, when dissolved in water or alkaline picrate solution, gave *immediately* the full color value of the creatinine content. This compound contained 1 mol of creatinine, 1 of picric acid, and 2 of sodium hydroxide. It was evidently the disodium compound of the red tautomer, or isomer, of creatinine picrate described in Paper I of this series (3). The corresponding barium salt has since been described by Anslow and King (4).

It seemed to be of interest to attempt to prepare the free acid from which the sodium compound that did *not* give Jaffe's reaction

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was derived. For this purpose, the alkaline reaction mixture was run into a slight excess of hydrochloric acid. The orange precipitate obtained was filtered out, washed with ice water, and dried *in vacuo*. As expected, it contained 2 mols of creatinine and 1 of picric acid (besides 1 or 2 mols of water) and did not give Jaffe's reaction until after it had been dissolved in dilute acetic acid.

It was observed as the alkaline reaction mixture was run into the excess of acid that there was no precipitate until most of the acid had been neutralized. The dicreatinine-picric acid compound was evidently soluble in excess of acid. This indicated the possibility of the existence of a combination of this new compound with acid.

In an attempt to isolate this, the alkaline reaction mixture was run into an excess of acetic acid. No precipitate was obtained. The clear liquid was run into alcohol. An orange precipitate was obtained. This was washed with alcohol and dried *in vacuo*. Analyses showed this to be not a compound with acid, but one with alkali.

It resembled the sodium compound previously described in that it did not give Jaffe's reaction directly. It differed in that it contained only $2\frac{1}{2}$ mols instead of 3 for each mol of picric acid and in that, when treated with concentrated sulfuric acid, it lost only one-ninth, instead of two-ninths of its total nitrogen. This might indicate that the compounds had larger molecules than had been supposed and that they contained 4 mols of creatinine and 2 of picric acid and, in the case of the sodium compounds, 6 or 5 mols of sodium hydroxide.

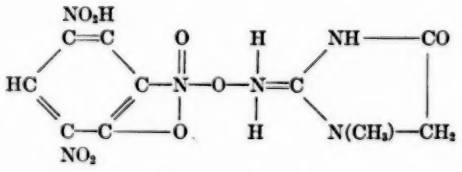
The solution of this new sodium compound was neutral to litmus. It was quite obvious, therefore, that the dicreatinine-picric acid compound was a strong acid and that a combination with acid could occur, if at all, only at a high concentration of hydrogen ions. Since alcohol seemed to be the most available precipitant, hydrochloric acid could not be used because sodium chloride would also be precipitated. Hydrobromic acid was substituted. The clear acid solution was run into absolute alcohol but there was no precipitation. However, when propyl alcohol was used, an abundant orange precipitate was obtained. This was filtered out, washed with propyl alcohol, and dried *in vacuo*,

over H_2SO_4. Analyses showed it to contain 2 molecules of creatinine, 1 of picric acid, 1 of hydrobromic acid, and 5 or 6 of water. It is true that no preparation gave figures that agreed precisely with this formula but it is quite obvious from the method of preparation that the compound is likely to be contaminated with the free dicreatinine-picric acid compound and with products of decomposition of this and the hydrobromide.

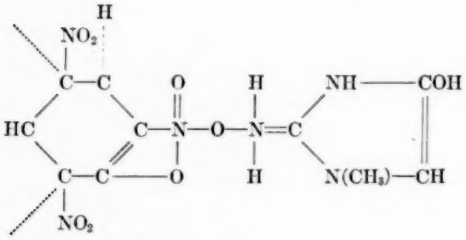
The compound is not very stable, even after drying. Thus a preparation that contained 36.5 per cent picric acid in June, was found to contain only 28 per cent in October. When moist, it is much more unstable. If this compound is suspended in water and heated, practically no picric acid can be precipitated by subsequent treatment with nitron nor can creatinine be precipitated by the addition of picric acid. Only if suspended in sodium or ammonium acetate solution and allowed to stand for several hours does the decomposition into the original constituents proceed at all smoothly.

Both the dicreatinine-picric acid compound and its hydrobromide differ from the compounds with sodium hydroxide in that no nitrogen is split off when they are dissolved in hot concentrated sulfuric acid. This would indicate a difference in structure.

A study of the behavior of substituted creatinines with alkaline picrate solutions led to the conclusion (5) that "the production of the red color . . . depends upon the formation of the enolic form of creatinine and upon the retention of the basic properties of the molecule." On the assumption that picric acid, in picrates, is in the *o*-quinone form, the formula for yellow creatinine picrate was written

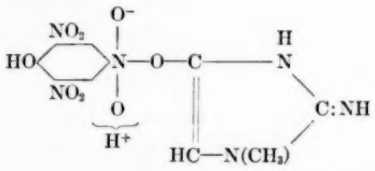


and the red tautomer, or isomer, was provisionally given the formula

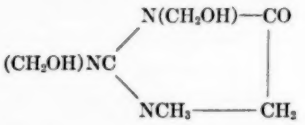


"The dotted lines indicate that the exact positions of these hydrogens and the disposition of the remaining valences of the carbon atoms are not known."

Anslow and King (4) have taken exception to this formula and have proposed one of their own:



But this formulation fails to explain why dimethylol creatinine



fails to give the reaction, nor why, neither the dinitrophenols nor trinitro-*m*-cresol gives colors with creatinine (and alkali) at all resembling that given by picric acid.

In their discussion they state, "It is of interest that two sodium salts, one containing 1 molecule of creatinine, 1 of picric acid, 2 of sodium hydroxide, the other, 2 molecules of creatinine, 1 of picric acid and 3 of sodium hydroxide have been prepared by Greenwald and fit in with the above formulation of the red acid. The presence of three nitro-groups in the picric acid would allow of the addition of at least two mols of creatinine in the way formulated above, and the compound formed would require 3 atoms of sodium as found by Greenwald."

Anslow and King seem to have failed to recognize that the second of these compounds, that which contains 2 mols of creatinine to 1 of picric acid, does not give Jaffe's reaction until after the compound has been decomposed by dilute acetic acid. If the formulation of Anslow and King were correct, the dicreatinine compound when treated with alkaline picrate solution as in the determination of creatinine might be expected to dissociate into the monocreatinine compound which has the intense red color of Jaffe's reaction. This is not the case. Moreover, the second creatinine cannot be combined in the same manner as Anslow and King assume the first to be, for 1 or 2 of the nitrogen atoms of the dicreatinine sodium or lead compounds are easily split off by treatment with concentrated acids. This is not the case with the red sodium compound containing equimolecular quantities of creatinine and picric acid. Moreover, since the free dicreatinine-picric acid compound and the hydrobromide do not lose nitrogen when treated with hot concentrated sulfuric acid, the nature of the combination of at least 1 of the nitrogen atoms cannot be the same in these as in the sodium or lead salts.

A number of possible formulæ might be suggested but it seems best to await a study of the products of the decomposition of the dicreatinine-picric acid compounds as well as the results of the attempted preparation of similar compounds containing (*a*) homologues of creatinine and (*b*) other nitrophenols.

EXPERIMENTAL.

The methods of analysis were those previously described (1) except that precipitation of picric acid was attempted only after the substance had been allowed to stand in dilute acetic acid (plus ammonium acetate in the case of the hydrobromide) for from 12 to 16 hours. Bromine was determined, gravimetrically, by precipitation with silver nitrate, either directly or in the filtrate from the picric acid determination, after precipitation of the excess nitron with nitric acid.

1. Compound of 2 Mols of Creatinine and 1 of Picric Acid.—To a solution of 4.52 gm. of creatinine in 100 cc. of water, there were added 6.84 gm. of creatinine picrate. 20 cc. of 6 N NaOH were then added. The temperature rose from 18 to 22°. After 15

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minutes, the mixture was placed in a freezing mixture and the temperature lowered to 0°. It was then run in a fine stream into 25 cc. of 6 N HCl contained in a beaker immersed in a freezing mixture. The maximum temperature during this process was 5°. The orange precipitate was filtered out and washed with ice water. After drying over H_2SO_4, *in vacuo*, this weighed 1.4 gm. The results of analyses are given in Table I (Preparation 98 A).

The filtrate from this precipitate became turbid as it was

TABLE I.

Composition of Different Preparations of Compounds of 2 Mols of Creatinine and 1 of Picric Acid.

Preparation No.	Total N.	Picric acid.	Picric acid N.	Mols creatinine per mol picric acid in reaction mixture.
	per cent	*per cent*	*per cent*	
78	25.7	47.0	8.62	3
81	26.5	48.3	8.86	3
86	26.1	48.0	8.81	5
98 A	26.4	48.2	8.84	3
98 B	25.6	47.0	8.62	
104	25.4	47.1	8.64	3
113	25.1*	46.3	8.50	3
118	25.5	46.7	8.57	5
Calculated for compound of: 2 mols creatinine, 1 picric acid, $1H_2O$.	26.6	48.4	8.84	
Same, with 2 mols H_2O.	25.7	46.6	8.56	

* When dissolved directly in hot concentrated sulfuric acid, 24.3 per cent.

diluted with the wash liquid. Addition of 6 cc. of 6 N NaOH produced a clear solution. 6 N HCl was then added until the mixture was distinctly acid to Congo red paper. For this purpose 4 cc. were required. The mixture was filtered and washed with ice water. After drying, this weighed 0.8 gm. It appears as Preparation 98 B in Table I.

Determination of Creatinine Content.—37.6 mg. of Preparation 81 were dissolved in a mixture of 10 cc. of H_2O, 15 cc. of 1 per cent

picric acid, and 5 cc. of 10 per cent NaOH. After 10 minutes, the mixture was diluted to 200 cc. and compared with 0.5 N $K_2Cr_2O_7$ set at 8.0 mm. The readings were 10.4, 10.2, and 10.2 mm., or the equivalent of 3.15 mg. of creatinine or 8.37 per cent.

23.8 mg. of the same preparation were allowed to stand overnight in 10 cc. of 1 per cent acetic acid. The next morning solution was not complete and the mixture was heated to boiling and then cooled. 10 minutes after the addition of 15 cc. of 1 per cent picric acid and 5 cc. of 10 per cent NaOH, the mixture was diluted to 500 cc. and compared with 0.5 N $K_2Cr_2O_7$ set at 8.0 mm. The readings were 7.4, 7.5, 7.5, and 7.3 mm., or the equivalent of 10.96 mg. of creatinine or 46.1 per cent. This is the calculated content for a compound of 2 mols of creatinine, 1 of picric acid, and 2 of water.

TABLE II.

Analyses of Compound of 2 Mols of Creatinine, 1 of Picric Acid, and 2.5 of Sodium Hydroxide.

Preparation No.	Total N.	Picric acid.	Picric acid N.	Na	C	H
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
70	20.3	38.9	7.12	10.1		
92	22.2	39.6	7.26	10.3		
94	22.1*	39.4	7.23	10.4	29.4†	4.11†
Calculated for composition of 1 mol picric acid, 2 creatinine, 2.5 NaOH, 2 H_2O.	22.1	40.1	7.37	10.07	29.4	4.11

* When dissolved in hot concentrated sulfuric acid, 19.9 per cent.

† For these analyses I am indebted to Dr. W. A. Jacobs.

2. Compound with 2.5 Mols of NaOH.—To a solution of 4.52 gm. of creatinine in 100 cc. of H_2O there were added 6.84 gm. of creatinine picrate and 20 cc. of 6 N NaOH. The temperature rose from 15 to 20°. After 10 minutes at 20°, the mixture was cooled to 0° and 10 cc. of glacial acetic acid were added. The mixture was then run in a fine stream into 1200 cc. of absolute alcohol. The orange precipitate was washed with 95 per cent alcohol and dried over H_2SO_4 *in vacuo*. It weighed 10.3 gm. After analysis, with the results given in Table II for Preparation 92, the remainder of the

material was dissolved in 100 cc. of water (the solution was neutral to litmus) and run into 1000 cc. of absolute alcohol. After being filtered and washed and dried *in vacuo*, the material was analyzed with the results given in Table II for Preparation 94.

Determination of Creatinine Content.—58.4 mg. of Preparation 94 were dissolved in a mixture of 10 cc. of water, 15 cc. of 1 per cent picric acid, and 5 cc. of 10 per cent NaOH. After 10 minutes, the mixture was diluted to 100 cc. and compared in a colorimeter with a solution containing 5 mg. of creatinine in 250 cc. and set at 20 mm. The readings were 26.4, 26.2, 26.2, and 26.2 mm., or the equivalent of 1.53 mg. of creatinine or 2.62 per cent.

25.1 mg. of the same preparation were dissolved in 10 cc. of water and 1 drop of acetic acid and the mixture was allowed to stand overnight. After the addition of the picric acid and sodium hydroxide the mixture was allowed to stand 10 minutes and was then diluted to 500 cc. The standard contained 5 mg. in 250 cc. and was set at 20 mm. The readings were 21.0, 21.2, 21.2, and 21.0 mm., or the equivalent of 9.48 mg. of creatinine or 37.8 per cent. The calculated amount is 39.6 per cent.

Recovery of Creatinine Picrate.—0.6012 gm. of the same preparation was dissolved in 30 cc. of water. 0.5 cc. of acetic acid was then added and the mixture set aside. The orange color gradually became yellow and in about an hour the solution became turbid. About 3 hours later, 0.5 gm. of picric acid, dissolved in a minimal quantity of hot water, was added. The solution was then placed in the ice chest overnight. The crystals that separated were filtered out and recrystallized. After drying, they weighed 0.3173 gm., melted at 217°, and were found to contain 66.0 per cent picric acid and 32.1 per cent creatinine. Calculated for creatinine picrate, 67.0 per cent picric acid and 33 per cent creatinine. The calculated yield of creatinine picrate is 0.732 gm. After correction for the solubility in the 100 cc. of mother liquor, 0.600 gm. was to be expected. Creatinine picrate seems to be more soluble in this liquid than in pure water. Colorimetric determinations indicated the presence of 0.117 gm. of creatinine in this solution, giving a total recovery of 0.220 gm. or 36.6 per cent.

3. Compound with Hydrobromic Acid.—To a solution of 4.58 gm. of picric acid in 125 cc. of boiling water, there were added 6.78

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gm. of creatinine. After cooling to 18°, 20 cc. of 6.51 N NaOH were added. The temperature rose to 23°. After 10 minutes, the mixture was cooled in a freezing mixture to —7° and was then run into 50 cc. of 6.51 N HBr at —10°. During this process, the temperature did not rise above 2° and at its close was 0°. The mixture was then run into 1000 cc. of *n*-propyl alcohol at —8°, with constant stirring. The mixture was kept at 5° for 30 minutes and was then filtered and washed with ice-cold propyl alcohol. After drying over H_2SO_4, *in vacuo*, the yield was 4.1 gm. This constituted Preparation 154. The combined filtrates were kept at 5°. An additional precipitate separated. This was filtered out, washed with propyl alcohol, and dried over H_2SO_4 *in vacuo*.

TABLE III.

Analyses of Compound of 2 Mols of Creatinine, 1 of Picric Acid, and 1 of Hydrobromic Acid.

Preparation No.	Total N.	Picric acid.	Picric acid N.	Br
	per cent	*per cent*	*per cent*	*per cent*
90	19.5	35.6	6.54	12.2
154	19.4*	36.5	6.70	12.4
156	19.8	40.0	7.34	12.2
Calculated for composition of 2 mols creatinine, 1 picric acid, 1 HBr, 5 H_2O.	20.13	36.60	6.71	12.77
Same, with 6 H_2O.	19.57	35.55	6.52	12.41

* When dissolved in hot concentrated sulfuric acid, 19.7 per cent.

The yield in this fraction was 3.1 gm. (Preparation 156, Table III).

Determination of Creatinine.—24.9 mg. of Preparation 154 were dissolved in a mixture of 10 cc. of H_2O, 15 cc. of 1 per cent picric acid, and 5 cc. of 10 per cent NaOH. After standing 10 minutes, the solution was diluted to 50 cc. and compared in a colorimeter with a standard containing 5 mg. of creatinine in a volume of 250 cc. and set at 20 mm. The readings were 21.2, 21.3, 21.2, 21.2, and 21.2 mm., or the equivalent of 0.94 mg. of creatinine or 3.8 per cent.

34.4 mg. of the same preparation were left in 10 cc. of a dilute sodium acetate solution overnight. The usual reagents were

added and, after 10 minutes, the solution was diluted to 500 cc. The standard contained 5 mg. of creatinine in a volume of 250 cc. and was set at 20 mm. The readings were 15.3, 15.3, 15.3, 15.2, and 15.2 mm., or the equivalent of 13.1 mg. of creatinine or 38.1 per cent. Calculated for the compound with 5 H_2O, 36.1 per cent; with 6 H_2O, 35.1 per cent.

Recovery of Creatinine.—1.064 gm. of Preparation 154 were ground with 1 gm. of $NaOAc \cdot 3H_2O$ in a small mortar. Upon the addition of 10 cc. of water, a clear red solution was obtained. This was transferred to a beaker containing 400 cc. of ice water and allowed to stand at 5° overnight. No crystals having separated, 0.5 gm. of picric acid was dissolved in a little hot water and added. Upon standing, crystals separated. These were filtered out and recrystallized twice. They melted at 215–217° and contained 66.0 per cent picric acid and 33.0 per cent creatinine. (Calculated for creatinine picrate, 67 per cent picric acid and 33 per cent creatinine.) The total yield was 0.34 gm. The calculated yield, without allowance for solubility of creatinine picrate, was 1.13 gm. The 160 cc. of mother liquor would be expected to contain about 0.21 gm. of creatinine picrate, so there is a possibility that 1 mol of creatinine had suffered an irreversible change. But the product of its transformation must have had about the same action on alkaline picrate solution as creatinine itself for colorimetric analyses indicated the presence of 0.27 gm. of creatinine, making with that contained in the crystals, a total of 0.38 gm. or 35.7 per cent which is close to the calculated value.

SUMMARY.

In the presence of an excess of creatinine and alkali, picric acid may combine with 2 mols of creatinine. The resulting compound has been isolated in the free state and in the form of its combination with (*a*) 3 mols of sodium hydroxide, (*b*) $2\frac{1}{2}$ mols of sodium hydroxide, and (*c*) 1 mol of hydrobromic acid. None of these compounds gives Jaffe's reaction for creatinine until after solution in dilute acetic acid-acetate buffer solutions.

The formulæ suggested by Anslow and King for the red isomer of creatinine picrate and for the dicreatinine-picric acid compound are considered to be unsatisfactory. The necessity for assuming

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quite different arrangements in (*a*) the red isomer, (*b*) the alkali salts of the dicreatinine compound, and (*c*) the free dicreatinine compound and its hydrobromide, is indicated.

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SUGAR ACTIVATION BY ALKALI.

I. FORMATION OF LACTIC AND SACCHARINIC ACIDS.

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It is well known that in alkaline solution reducing sugars exhibit an instability and behavior which resembles in some important respects the changes accompanying the utilization of sugars by living cells. They develop strong reducing intensity and become autooxidizable, are to a considerable extent interconvertible, are polymerized and depolymerized, and in the absence of oxidizing agents are converted in part into lactic acid with the intermediate formation of methylglyoxal. The fact that each of these phenomena has its counterpart in carbohydrate metabolism, gives to the evident activation of sugars by alkali a special biochemical interest, besides the fascination of the reactions on purely chemical grounds.

The numerous investigations of the complex transformations which sugars undergo under the influence of cells or tissue extracts on the one hand and of alkali on the other have been frequently interpreted and reviewed (1–4) with the object of deciphering the path and sequence of the reactions involved. In both fields a central problem has been the search for the "active" forms of the sugars to which the characteristic instability may be attributed. The trend of recent conceptions is toward the choice of hypothetical ring structures as the "active" sugars. See Levene's review (2).

A survey of the subject seems to us however to point to the

* The experimental data are taken in part from a dissertation presented by T. E. Friedemann in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Washington University, 1923.

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conclusion that *in alkaline solution* the instability of sugars is primarily the consequence of the formation of salts of the sugars acting as weak acids, a view advocated by Mathews (5) in connection with sugar oxidation and emphasized particularly by Nef (6, 7). Monobasic salts of glucose have long been known (8), and the acidic dissociation constants of various sugars have been determined (9). The first "active" forms of the sugars appear to be sugar ions, and their formation is probably the enabling act so to speak in each of several types of sugar transformation under the influence of alkali. The phenomena of mutarotation, involving isomerization of carbon atom 1 is due according to Lowry (10) to ionization of the sugar hydrate, while the data of Euler and associates (11) appear to show that the catalytic effect of acids and bases is due to salt formation and consequent increase in concentration of sugar ions. Somewhat similarly, the rate of loss of optical activity resulting from the Lobry de Bruyn aldose-ketose transformation, which involves isomerization of carbon atoms 2 and 3, is shown by the work of Groot (12) with glucose to be proportional not to pH (Michaelis and Rona (13)) but rather to the fraction of the sugar present in the form of alkali salt. Although having widely different velocities, both these transformations appear to be the spontaneous rearrangements of unstable sugar ions.

In this paper we present data concerning the relation of degree of alkalinity to a third type of non-oxidative transformation of sugars; namely, saccharinic acid and in particular lactic acid formation. We have attempted to correlate these reactions also with salt formation. The data considered here represent *final yields* of total saccharinic and lactic acids; in another paper the *rates* of reaction will be reported. After our experiments were undertaken and in large part completed, a series of papers by Evans and associates (14–18), covering somewhat similar experimental ground, appeared. Their results will be cited in relation to our own. Evans has recently published a summary of (19) this work. See also the recent studies of Bernhauer and associates (20–23) and Fischler and coworkers (24). We shall first review briefly the main facts and theories concerning the production of saccharinic acids.

Formation of Saccharinic Acids.

From the investigations by Kiliani (25) and others, especially Nef and his pupils (6, 7, 26–29), there have been isolated and identified as the chief products of the anaerobic destruction of hexoses by strong alkali, besides sugar resins and racemic lactic acid, *dl*-1,3-dihydroxybutyric acid, and a series of optically active 6-carbon acids, the meta-, iso-, and parasaccharins. The main products are thus C_3, C_4, and C_6 acids of the same empirical formulæ as the sugars $(CH_2O)_n$, the first two being racemic. In 8 N alkali about the same amounts of lactic acid are formed from glucose and fructose but less from galactose, which gives relatively more of the 4- and 6-carbon acids, the latter being different in the case of galactose from the 6-carbon acids of glucose and fructose. From the pentoses were obtained smaller amounts of lactic acid, the same *dl*-1,3-dihydroxybutyric acids, and optically active 1,3,4-trihydroxyvalerianic acids. With more dilute caustic alkali Nef stated that there is formed in all cases "ein furchtbares Gemisch aller denkbaren Saccharinsäuren, mit drei, vier, funf und sechs Kohlenstoffatomen" ((6) p. 9). He noted that the particular saccharinic acids formed depend upon the strength of alkali (see also Upson (28)).

Based on structural theory, on the sugars formed in weak alkali (the Lobry de Bruyn transformations), on the structure of the various saccharinic acids formed in strong alkali, and the products of oxidation, it will be recalled that Nef formulated a detailed general theory of the reactions, as proceeding through a series of intermediates, the 1,2-, 2,3-, and 3,4-dienols. Diagram 1, representing the relations and sequence of events, will aid in the following discussion.

The idea as to the formation of intermediate dienols, an extension of explanations advanced by Lobry de Bruyn and van Ekenstein (30) by Fischer (31) and by Wohl and Neuberg (32), supplies the means of passing from aldoses to 2- and 3-ketoses. In alkali carbonate or quite dilute hydroxide with large excess of sugar the reactions are practically limited to these transformations, involving isomerization of carbon atoms 2 and 3 without much saccharinic acid formation. With sufficiently dilute alkali and low temperature they may be almost wholly confined to carbon atom 2 with aldoses predominating. See the recent results of Wolfrom and

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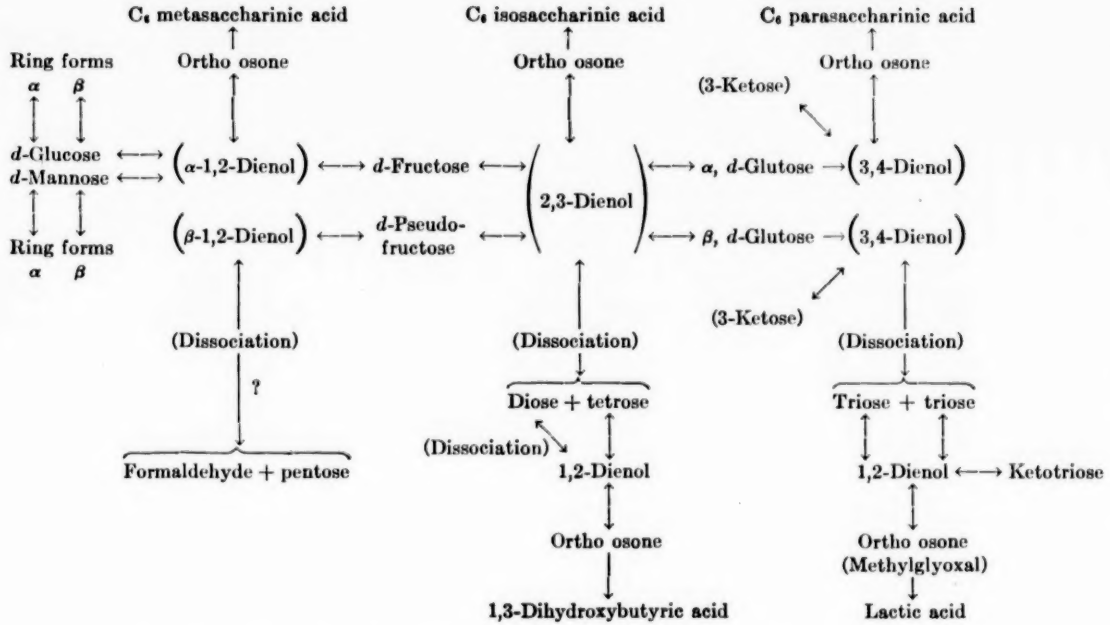
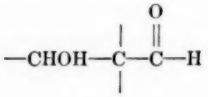


DIAGRAM 1. Outline of relations among sugars in alkalinic solution after Nef (*d*-glucose series).

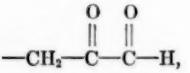
Lewis (33), and Greene and Lewis (34). At similar low alkalinity slow polymerization of lower to higher sugars also occurs. With higher alkalinity or temperature the aldoses decrease in proportion and non-fermentable sugars of low optical activity appear, among which are a second 2-ketose and two 3-ketoses (from the glucose series *d*-pseudofructose, α-, and β-*d*-glutoses). Although to a considerable degree reversible as regards aldoses and 2-ketose (glucose-fructose-mannose), the 3-ketose formation appears to be irreversible; at sufficiently high alkalinity it passes readily to saccharinic acid (Lobry de Bruyn and Nef; see also Benedict, Dakin, and West (35) and Spoehr and Wilbur (36)).

Nef was apparently of the opinion that *enolization* may occur *without* salt formation ((7) p. 225 and 241), being due to loss of water by splitting hydroxyl from the aldehyde or ketone hydrate and H from an adjacent carbon. But since alkalinity induces enolization, perhaps the enol *is* a salt. As pictured by Nef's pupils, Spoehr ((3) p. 18) and Woodyatt ((4) p. 659), the salt is that of the hydrate hydroxyl which loses OM and H (as OH and H above) to *form* the dienols. Wolfrom and Lewis (33) disagree with the view that water (or MOH) is lost (and added in the reverse direction) on the ground that tetramethylglucose and tetramethylmannose would thus form hemiacetals which should lose CH_3OH and yield 3,4,6-trimethylfructose, which they find not to occur. Interconversion of the two methylated aldoses does take place, and an intermediate is formed. They picture enolization therefore as simple migration of H (as H^+ ion) from carbon atom 2, which amounts to attributing to it some acidic property. Fischer (31) regarded sodium glucosate as a salt of 1,2-dienol. Evans writes ((19) p. 309) the dienols as dibasic acids.

The limited equilibrium existing among the aldoses, ketoses, and intermediate dienols, is disturbed at higher alkalinity by two other reactions, both of which lead to saccharinic acids. According to Nef's theory a different acid is derived from each aldose and ketose present, as a result of salt formation and rearrangement first into respective orthodiketo forms, the "ortho osones," the best known example of which is methylglyoxal. He supposed that the metal (M) takes the place of H of the hydroxyl group *adjacent* to the carbonyl group. By loss of MOH the salt becomes



which rearranges to



which under the influence of alkali adds MOH to give $-CH_2$-CHOH-COOM. The meta-, iso-, and parasaccharinic acids are thus derived through benzylic acid rearrangements from aldoses, 2-, and 3-ketoses respectively ((27) p. 301). A somewhat different derivation of the saccharinic acids is proposed by Evans and Benoy (cited by Evans and associates (14) p. 2675), who relate them directly to the 1,2-, 1,3-, and 3,4-dienols, which thus become the immediate precursors of both cleavage fragments and ortho osones. This relation is incorporated in Diagram 1.

The other reaction is the spontaneous dissociation (theoretically reversible) of each of the dienols into hydroxymethylene fragments, nascent aldoses (R-C-OH) which, having properties of free radicals, are readily oxidized, or in the absence of oxidizing agents, are again enolized and converted to corresponding saccharinic acids or are polymerized. Lactic acid is the metasaccharinic acid derived by dissociation of 3,4-dienol (of the hexoses) and conversion of the aldo triose via its ortho osone, methylglyoxal. The reaction dienol to ortho osone is also apparently reversible, in view of the recent observations by Bernhauer and Görlich of the formation of triose and hexose from methylglyoxal (22) at low alkalinity. The 1,3-dihydroxybutyric acids are similarly derived from tetrose, formed by dissociation of hexose 2,3-dienol. Nef found no evidence for dissociation of hexose 1,2-dienol except on oxidation. The liability of the dienols to rupture increases in the order 1,2-, 2,3-, 3,4-, the last disrupting rapidly. The dissociation of the dienols Nef thought to occur spontaneously, independent of salt formation. It seems more probable that the enols exist mainly in the form of salt or ion, possibly of dibasic acids as suggested by Evans. Fischler (24) regards the splitting of hexose to triose as the rupture of a salt of the hydroxyl of carbon atom 4.

From the above account it will be noted that ionization or salt formation has been demonstrated or hypothecated in connection with (1) mutarotation, (2) enolization with aldose-ketose conversion, (3) polymerization of low to higher sugars and dissociation of sugars or their dienols, (4) rearrangement to ortho osones and saccharinic acids, and (5) sugar oxidation. In these several reactions the mobility or dissociation of *different* H atoms (protons) appear to be involved, a fact which suggests that the sugars may behave as di- or polybasic acids.

In the experiments here reported it was desired to learn the extent of variations in the *final amounts* of the several saccharinic acids formed from various sugars at different levels of alkalinity. Because of the impossibility at present of separate determination of individual acids we resorted to the simple plan of determining the total by titration of the base bound, and of determining lactic acid by a rapid oxidation method (37) which was at first thought to be fairly specific for that substance. The amount of total acids less lactic acid gives approximately the sum of all others, the C_4 and C_6 acids.

Since the validity of the data depends upon the reliability of the method for determination of lactic acid in the presence of sugars and of other products of their destruction, this point was separately investigated. According to the results of that work (38), we conclude that the lactic acid figures here reported for glucose, fructose, mannose, and trioses are correct within about 5 per cent. Some of the results may be to this extent higher than the true values, because of plus errors due mainly to 1,3-dihydroxybutyric acid, which upon analysis by the method used is found to be equivalent to about one-tenth as much lactic acid. Lactic acid accompanied by an equal amount of this acid would therefore give results about 10 per cent too high. But pure lactic acid gives results about 2 to 4 per cent too low, which thus tends to balance plus errors from moderate amounts of the 1,3-hydroxybutyric acid. Interference by other saccharinic acids is believed to be negligible. Residual reducing sugars, resins, and much of the higher acids are removed by copper-calcium hydroxide precipitation or are left behind by ether extraction. With glucose and fructose we have been able to isolate after ether extraction amounts

of analyzed zinc lactate which agree fairly well with results by the oxidation method.

With galactose, arabinose, and xylose, however, the yield of zinc salt is lower than corresponds to results by the oxidation method, and it is probable that the latter are too high with these sugars because of the much larger amounts of 1,3-dihydroxybutyric acid formed from them (Nef). On the other hand the results by isolation of zinc salt are undoubtedly too low, because of incomplete crystallization in the presence of much other acids. Our "lactic acid" results from these three sugars are so designated in order to indicate their uncertainty. A series of experiments was conducted also with glycolaldehyde, which after decomposition by alkali followed by ether extraction or copper-lime precipitation yields by oxidation moderate amounts of apparent lactic acid. We did not succeed, however, in isolating zinc lactate from the solutions. Nef (and more recently Evans (19)) failed to isolate lactic acid from the decomposition of this sugar, but did find 1,3-dihydroxybutyric acid. It is therefore doubtless this substance which gives acetaldehyde on oxidation, and thus appears to be lactic acid by this method. The data from this sugar are therefore omitted.

Procedure.

Known amounts of the sugars, dissolved in air-free water, were added to known amounts of KOH solution (previously boiled and cooled) in volumetric flasks, the contents being then diluted to the mark with freshly boiled and cooled water. After rapid mixing exposure to air was minimized by covering the solution with a layer of mineral oil or by replacing air over the liquid by nitrogen. This precaution is very important in order to avoid oxidation of the strongly reducing intermediates. The tightly stoppered flasks were allowed to stand for days or months until it was thought, from other data, that the reactions had come about to an end. Control experiments containing the same concentrations of alkali without sugar were treated in the same way as blanks for titration of total base.

The amounts of sugar and of alkali were so chosen that the alkali would always be present in large excess over that required to

neutralize the acids formed, in order that the reduction in alkalinity due both to the sugar[1] and acids formed would not introduce too large a variable during the course of the reactions. With the more dilute alkali solutions the concentration of sugar was also small. The final alkalinity in each of the experiments presented may be learned by subtracting the product of total acid times mM of sugar taken, from the initial alkalinity. For example, (Table I) starting with 0.02 N KOH and 5 mM glucose had at the end $20 - (5 \times 1.59) = 12.05$ or 0.012 N KOH. In others the reduction of alkalinity was much less: from 0.04 to 0.031 N, from 0.10 to 0.083 N, from 0.25 to 0.232 N, and so on.

At the conclusion of the reaction period 5 to 20 cc. aliquots of the solution were measured into 100 cc. of cold water and titrated with 0.1 N HCl to phenolphthalein for decrease of free base (total acid formed). Other aliquots were neutralized and after appropriate dilution were analyzed for residual reducing sugar by the Shaffer-Hartmann method (39), and for lactic acid by oxidation to acetaldehyde (37) after ether extraction or after precipitation by copper and lime, or both.

The sugars used were the following: glucose, Bureau of Standards and Eastman; fructose, Special Chemicals Company, and

[1] The following simple experiment demonstrates the base-binding power of sugars. To each of three test-tubes add the same amount of water (10 cc.) and of a dilute solution of indigo carmine (acid-base indicator in the range pH 12 to 14); in one tube dissolve about 0.5 gm. of glucose, and to this and one other tube add 1 cc. of N NaOH. In 0.1 N NaOH (pH about 13) the indigo assumes a yellow-green color, while in the tube containing the same concentration of NaOH in the presence of about 0.3 M glucose, the color remains blue; the base is neutralized by the acidic glucose to the extent that the solution has a pH below the zone of color change of the indicator. The same experiment gives a like result with stronger solutions of alkali if the sugar concentration be likewise increased. After a few minutes the color of the sugar tube changes from blue to carmine to yellow, due to the *reduction* of the dye, a phenomenon which follows, but is quite distinct from the *instantaneous* change of color due to the formation of the sugar salt. The reduction of the dye indicates activation of the sugar as a reducing agent. If an indicator such as phenolphthalein, changing color at lower pH, be substituted and the solution made just alkaline to that indicator, the addition of glucose gives no indication of its acidic property, showing that a greater alkalinity is required to call forth a perceptible dissociation.

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Kahlbaum; mannose and galactose, Special Chemicals Company; xylose and arabinose, Kahlbaum; dihydroxyacetone[2]

TABLE I.

Saccharinic Acid Formation from Hexoses at 37–40°.

Results are expressed as mols per mol of hexose.

Initial concentration.		Total saccharinic acids.				Lactic acid.				Other acids by difference.			
KOH	Hexose.	Glucose.	Mannose.	Fructose.	Galactose.	Glucose.	Mannose.	Fructose.	Galactose.	Glucose.	Mannose.	Fructose.	Galactose.
M	*mM*												
mNa$_2$CO$_3$	40	1.29				0.17				1.12			
0.02	5	1.59	1.63	1.58	1.49	0.55	0.57	0.54	0.40	1.04	1.06	1.04	1.09
0.04	5	1.69	1.75	1.75	1.65	0.72	0.72	0.70	0.46	0.97	1.03	1.05	1.19
0.10	10	1.69	1.69	1.69	1.49	0.84	0.86	0.86	0.52	0.85	0.83	0.83	0.97
0.25	10	1.83	1.82	1.86	1.56	1.04	1.04	1.05	0.53	0.79	0.78	0.81	1.03
0.50	50	1.67		1.70	1.41	1.06		1.06	0.48	0.61		0.64	0.93
1.0	50	1.71		1.76	1.42	1.13		1.16	0.44	0.58		0.60	0.98
2.0	50	1.64		1.68	1.32	1.14		1.20	0.43	0.50		0.48	0.89
5.0	50					1.05		1.11	0.43				
9.2–10.0	50					1.08	1.01	1.13	0.57				

TABLE II.

Effect of Alkali Concentration on Total Acid and Lactic Acid Formation from Anaerobic Destruction of Pentoses at 37–42°.

Initial sugar concentration 60 mM per liter.

Initial KOH concentration.	Mols per mol of sugar.					
	Total acid.		Lactic acid.		Other acids.	
	Xylose.	Arabinose.	Xylose.	Arabinose.	Xylose.	Arabinose.
M						
0.5	1.27	1.26	0.49	0.45	0.78	0.81
1.0	1.29	1.29	0.54	0.47	0.75	0.82
2.0	1.30	1.30	0.51	0.48	0.79	0.82
5.0			0.48	0.51		
9.0			0.55	0.77		

The oxidation method by which the lactic acid data were determined is of doubtful reliability with products of pentoses.

("oxantin"), Farbwerke and Hoechst; glyceraldehyde, prepared by the Wohl and Lange method as modified by Witzemann (40).

[2] The "oxantin" used in these experiments was generously supplied to us by the Mallinckrodt Chemical Company.

The purity of the sugars was checked when possible by optical rotation, moisture, and ash; trioses, by the acid formed on oxidation by H_2O_2 in alkaline solution.[3]

Data showing the amounts of total acid and of lactic acid formed from the anaerobic destruction of glucose, fructose, mannose, and galactose in from 0.02 to 10 N KOH at about 40° are given in Table I. Data for arabinose and xylose are given in Table II and for glyceraldehyde and dihydroxyacetone in Table III. The results represent approximately maximum yields, the solutions having stood for periods of 2 months or longer. When analyzed, the reducing power had almost disappeared (less than 4 per cent remained), and it is assumed that the reactions had come to an end. Controls in some cases standing for much longer periods showed little or no additional change. A slight residual reduction is always found, even after many months. On longer standing exposed to air the reducing power and coincidentally the brown color of the solution decrease or disappear, but without any demonstrable further increase of lactic acid.

Effect of KOH Concentration.

Total Acids.—From the results it appears that the amount of *total* acids formed from glucose, fructose, and mannose is practically the same and is to a considerable degree *independent of the initial alkalinity* (from 0.02 to 2.0 N KOH), provided the alkali hydroxide is present in excess, as was the case in these experiments. At the lower alkalinity, M Na_2CO_3, the total acid formed from glucose was distinctly lower.[4] In another paper it will be shown that great differences exist in the *rates* of total acid formation from these three sugars at the same alkalinity, but these differences are not apparent in the *final* yields. The observed variations in amounts of acid are not regular and are perhaps within experimental errors.

[3] Method and results to appear in a later paper.

[4] In this experiment, 0.04 M glucose in M Na_2CO_3, the residual reduction after 4 days at 65°, corresponded to 5.3 mM of glucose. The calculations are based on the assumption that 34.7 mM of sugar were destroyed. If the remaining sugar were 2-ketoses of lower reducing power (35) toward the copper reagent used, the yields of acid per mol of sugar destroyed would be correspondingly increased.

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TABLE III.

Trioses. Effect of Alkali Concentration on Yield of Total Acid and Lactic Acid from Anaerobic Destruction at 37–42°.

KOH, initial concentration.	Sugar, initial concentration.	Mols per mol of triose.		
		Total acid.	Lactic acid.	Other acids.
Dihydroxyacetone.				
M	*mM per l.*			
0.02	10	0.78	0.25	0.53
0.04	10	0.95	0.32	0.63
0.10	10	0.75	0.40	0.35
0.25	10	0.71	0.53	0.18
0.50	10		0.67	
1.00	10		0.82	
2.00	10		0.90	
5.00	10		0.93	
10.00	10		0.95	
0.025	20		0.17	
0.050	20		0.25	
0.10	20		0.30	
0.25	20		0.42	
0.50	20		0.52	
1.00	20		0.65	
2.50	20		0.81	
5.0	20		0.86	
10.0	20		0.82	
0.25	40	0.823	0.328	0.495
0.50	40	0.855	0.43	0.425
1.00	40	0.95	0.60	0.35
1.00	40		0.593	
1.00	80		0.514	
0.50	100	0.865	0.435	0.43
1.00	100	0.913	0.513	0.40
2.00	100	0.91	0.657	0.253
5.00	100		0.827	
10.00	100		0.810	
1.00	160		0.472	
Glyceraldehyde.				
0.02	9.4	0.82	0.24	0.58
0.04	9.4	0.93	0.31	0.62
0.10	18.7	0.89	0.39	0.50
0.25	18.7	0.94	0.48	0.46
10.00	32.7		0.74	

The amount of total acid formed from glucose, mannose, and fructose (1.6 to 1.8 cc. of N per mM) and the somewhat smaller amount from galactose (1.3 to 1.6 cc. of N per mM) show that a portion of the sugar molecules, but not all, are ruptured to give more than 1 molecule of acid, even in 0.02 N KOH. The amount from pentoses is slightly less per mol than from galactose, indicating less rupture of the chain into two acid-forming fragments than occurs in the glucose series. The total acid formed from the two trioses is substantially the same, and, within the range of

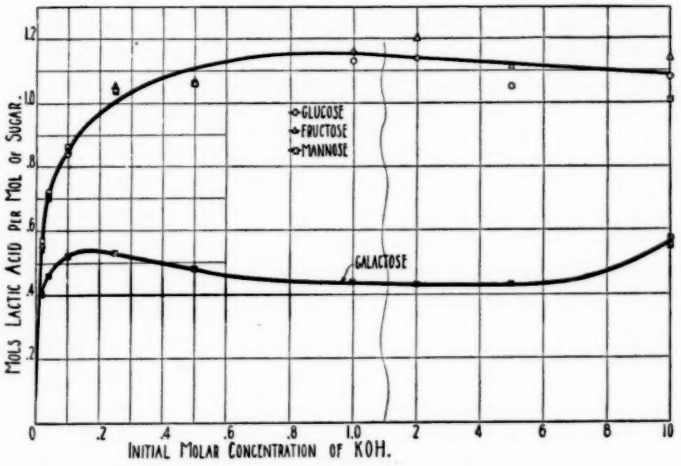

FIG. 1. The effect of the alkali concentration on the yield of lactic acid from glucose, fructose, mannose, and galactose at 37.5°.

alkalinity up to 0.5 N, the yield from 2 mols of triose *equals that from 1 mol of glucose.* This fact accords with the view, to be mentioned presently, that in the lower range of alkalinity the trioses are polymerized to hexose before being transformed to saccharinic acid.

Lactic Acid.—In marked contrast with the approximate constancy of the total acids, their distribution between lactic and other saccharinic acids varies with the alkalinity. With low alkali concentration the amount of lactic acid formed is small and the proportion of other acids is correspondingly great. As the

alkalinity is increased the proportion appearing as lactic acid increases, the ratio changing for glucose, mannose, and fructose from about 1 part lactic to 2 of other acids at 0.02 N alkali to about 2:1 at N alkali or above, the maximum yield of lactic acid being slightly over 1 mol per mol of hexose. With galactose, the rise of "lactic acid" (see p. 352) with alkalinity is less marked, reaching a maximum of about 0.5 mol per mol of hexose, which again indicates that the galactose chain (dienols) is less readily

TABLE IV.

Relation of Alkalinity to Yield of Lactic Acid.

Initial KOH.	Log $(OH)_a$ (activity 25°).	Log $(OH)_c$ (conductance 18°).	Final yield of lactic acid per mol of hexose or 2 mols of triose.					
			Glucose series av.	Dihydroxyacetone.				Glyceraldehyde.
				10 mM.	20 mM.	40 mM.	100 mM.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
M			*M*	*M*	*M*	*M*	*M*	*M*
0.02	−1.76	−1.72	0.55	0.50				0.48*
0.025	−1.67	−1.63			0.34			
0.04	−1.48	−1.43	0.72	0.64				0.62*
0.05	−1.39	−1.34			0.50			
0.10	−1.11	−1.04	0.86	0.80	0.60			0.78†
0.25	−0.74	−0.66	1.04	1.06	0.84	0.65		0.96†
0.50	−0.44	−0.38	1.06	1.34	1.04	0.86	0.87	
1.0	−0.13	−0.106	1.14	1.64	1.30	1.20	1.03	
2.0	+0.23	+0.13	1.17	1.80			1.31	
2.5	+0.375	+0.196			1.62			
5.0	(+0.87)	+0.35	1.08	1.86	1.72		1.65	
10.0	(+1.30)	+0.30	1.07	1.90	1.64		1.62	1.48‡

* 9 mM triose concentration.

† 19 mM triose concentration.

‡ 33 mM triose concentration.

broken than in the case of the three other hexoses. The results from pentoses are similar to those from galactose.

The yield of lactic acid (as well as total acids) at *any single alkalinity* is, within the limits of experimental error, the *same for each of the three hexoses*, glucose, fructose, and mannose, but quite different for galactose (Fig. 1). The similarity of the results with the first three sugars is in keeping with their relationship through the 1,2-dienol, and confirms the observation by Nef ((6) p. 89)

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and Evans ((19) p. 299).[5] It may be noted that 1 equivalent of KOH is not sufficient for maximum conversion to lactic acid.

The maximum yield of lactic acid from the hexoses, determined by the oxidation method, is somewhat greater than the amounts isolated by others as zinc salt.[6]

The trioses, glyceraldehyde and dihydroxyacetone, yield under similar conditions very nearly the same amounts of lactic acid, in confirmation of Evans and Cornthwaite (16). (The quantitative discrepancies between the results of Evans and our own with trioses are noted below.)

On comparing the lactic acid production from the trioses with that from glucose (or fructose or mannose) we encounter a remarkable relation. The data are given in Table IV and in Fig. 2.

[5] The lactic acid yields reported by Evans and coworkers from these hexoses are, however, quantitatively very different from our own, especially at the lower range of alkalinity. In 0.5 M KOH at 50° they find, for example, only 0.08 mol per mol of fructose (17) and 0.24 mol per mol of mannose and glucose (18) compared with our yield of about 1.04 mol in 0.5 M KOH. At lower alkalinity the discrepancy is still greater, at higher alkalinity less. The difference is due in large part to the high sugar concentration (0.5 M) used by Evans and the consequent initial decrease of alkalinity through binding of base by sugar. This point will be referred to also in connection with the results by these workers with trioses.

[6] Nef states ((6) p. 89) that from 100 gm. of glucose, fructose, or mannose there are formed in strong alkali 40 to 45 gm. of lactic acid. This is the equivalent of 0.8 to 0.9 mol per mol of hexose. The amounts recorded in his protocols are, however, less, from 40 gm. of glucose and fructose 16.4 and 17 gm. of zinc lactate ((26) p.326) or about 0.5 mol. Meisenheimer (41) and Oppenheimer (42) obtained from glucose and fructose from 0.6 to 1.0 mol of lactic acid per mol of hexose. Friedemann ((38) p. 80) obtained from glucose (1.1 mol in 3.5 N NaOH) 0.66 to 0.85 mol isolated as zinc salt, while the oxidation method on the same solution gave 0.86 mol per mol of hexose. Evans, Edgar, and Hoff (14) record a maximum of about 40 per cent conversion of glucose to lactic aid in 7 N KOH at 50°—or about 0.8 mol (isolated as zinc salt). Evans and O'Donnell (18) found approximately the same amounts of lactic acid from glucose, fructose, and mannose decomposed at 75° in KOH, the yield rising to a maximum of 35 per cent or 0.7 mol with rising alkalinity. At 50° glucose yielded 1 mol of lactic acid in 7 M KOH. From the decomposition of galactose Nef (25) and Meisenheimer (41) isolated about 15 per cent of lactic acid (about 0.3 mol), and much more 1,3-dihydroxybutyric acid (Nef) than from glucose; while Evans, Edgar, and Hoff obtained less than 2 per cent of lactic acid. Wolf (23) reports yields of lactic acid amounting to 70 per cent or more of theory from sucrose by heating with 1 or 2 mols of CaO to 200° in an autoclave.

Lactic acid yields, expressed in mols from 1 mol of hexose or from the equivalent 2 mols of triose, are plotted against logs of initial millimolar KOH concentration. In the lower range of alkalinity up to about 0.1 to 1.0 N KOH, depending on triose concentration, the trioses yield somewhat *less* lactic acid than does glucose, but with greater alkalinity the trioses overtake the hexoses and

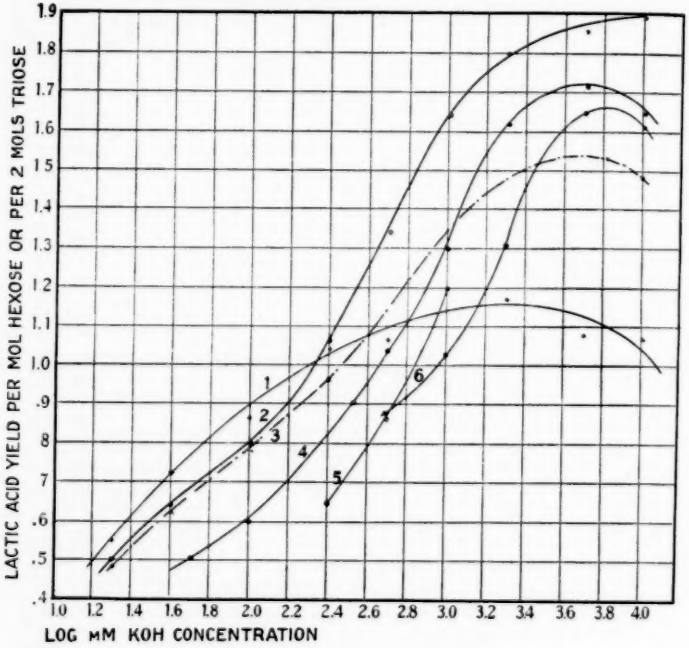

FIG. 2. Relation of initial alkali (KOH) concentration to yield of lactic acid from glucose and trioses. Curve 1 is for glucose; Curve 2, dihydroxyacetone 10 mM; Curve 3, glyceraldehyde; Curve 4, 5, and 6, dihydroxyacetone 20, 40, and 100 mM respectively.

approach quantitative conversion into lactic acid. The conversion is more nearly complete the higher the alkali concentration (though note that the curves dip downward at 10 N KOH) and the *lower* the triose concentration. It will be noted that the curve representing lactic acid yield from the series of 10 mM dihydroxyacetone

(and glyceraldehyde, 9 mM), parallels closely the glucose curve up to about 0.1 N KOH. Within this zone the yield of *total* acids is also substantially the same from the trioses and hexoses. These facts suggest that *in this range* of alkalinity the triose is converted more or less completely to hexose, and by this round about process yields approximately the same small portion of lactic acid as the hexoses (together with larger amounts of the C_4 and C_6 saccharinic acids). That triose *is* condensed to hexose in dilute alkali was shown by Fischer and Tafel (43). Above an alkalinity of 0.1 N KOH the 10 mM curve turns sharply upward and reaches 95 per cent of the theoretical lactic acid in 10 N KOH. At alkalinities corresponding to the rising slope of the curve hexose formation is suppressed, doubtless by a more rapid *direct* conversion of the triose to lactic acid. Although reversible equilibria are doubtless involved and influenced by alkalinity, it cannot be assumed that the high lactic acid yield from trioses is due merely to a displacement of the equilibrium, hexoses $\longleftrightarrow$ trioses, to the right by high alkali, because the yield much exceeds the maximum obtainable from hexoses. When once the 6-carbon chain is formed, its rearrangement to C_6 acids (without splitting) and rupture of the 2,3-dienol both intervene to reduce the yield of lactic acid.

The curves for 20, 40, and 100 mM dihydroxyacetone are less complete, but seem to fall in the same family and to differ by having the upward inflection spread out over wider zones, at progressively higher alkalinity, and by reaching progressively lower maxima.

Influence of Sugar Concentration.

To illustrate the influence of sugar concentration Fig. 3 has been prepared by plotting the data on the yield of lactic acid (per mol of triose) against concentrations of dihydroxyacetone. The curves show that at any given alkalinity the lactic acid yield tends to approach the theoretical at zero sugar concentration.

A similar effect of sugar concentration is shown for glucose by the following experiments. Varying quantities of glucose were added to air-free NaOH solutions of 3 to 6 N final concentration. In all cases the concentration of alkali was greatly in excess of the sugar, and the effect of change of alkali concentration may perhaps

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be ignored. After allowing sufficient time for approximately complete destruction, the lactic acid content and the relative amount of color were determined, the latter by dilution to equal initial *sugar* concentration and reading in a colorimeter. The results are shown in Table V. The data show a decline of lactic acid with increasing sugar concentration, and a reverse relation to color production, the effects becoming marked when the sugar concentration is molar and above.

The same effect may be demonstrated also with alkali-earth hydroxide. Varying amounts of glucose were placed in 250 cc. volumetric flasks, and the volume was brought to the mark by a 20 per cent suspension (previously boiled and cooled) of $Ca(OH)_2$.

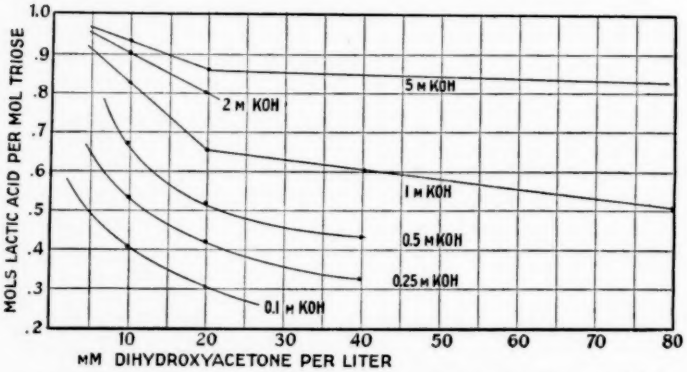

FIG. 3. Relation of triose (dihydroxyacetone) concentration to yield of lactic acid.

The tightly stoppered flasks were placed in a water bath at 37.5° and were frequently shaken during the first 12 hours. (The flasks were very nearly filled.) To insure complete reaction the flasks were allowed to react 18 days at 37.5°, and were then maintained at 65° for 1 week, with occasional shaking. The results, given in Table V, show a progressive decrease of lactic acid with increasing sugar concentration. Here the effect is in part due to reduction of initial alkalinity by added sugar, though excess $Ca(OH)_2$ was present in all flasks.

Nef obtained only very small amounts of lactic acid from glucose destroyed by $Ca(OH)_2$. His observation was due to the high

sugar concentration used as well as to incomplete separation of zinc lactate. With low sugar concentration, saturated $Ca(OH)_2$ (about 0.03 N) forms nearly as much lactic acid from glucose as does an equal alkalinity of alkali hydroxide.

There are doubtless several factors concerned in this effect of sugar concentration. One is the decrease of initial alkalinity due to base bound by sugar (before saccharinic acid formation). This decrease of alkalinity is, of course, greater with increasing

TABLE V.

Influence of Sugar Concentration on Lactic Acid Yield. Glucose.

Temperature 37–65°.

Initial glucose concentration.	Initial NaOH concentration.	Lactic acid yield per mol of glucose.	Relative color of solutions per mol of sugar.	Excess $Ca(OH)_2$.	
				Glucose concentration.	Lactic acid yield per mol glucose.
*m*M	*M*	*M*	*M*	*m*M	*M*
50	3.0	1.1	1.0	20	0.83
100	5.0	1.05		50	0.67
				100	0.45
550	5.0	1.01	1.4	250	0.31
1110	3.0	0.83		500	0.22
2000	6.0	0.79			
2600	6.0	0.74	5.4		
3400*	17.0+	0.46	22.0		

* 50 gm. of glucose, 60 gm. of powdered KOH, and 20 cc. of 18 M KOH were stirred. The reaction began immediately and was very violent. The volume of liquid at the end of the reaction was 82 cc. The temperature of the reaction was about 145°. It is included in this table to show the relation between the lactic acid yield and the formation of colored resin and products.

sugar concentration; but it is unlikely that this factor is very important in our experiments, in which the KOH was in large excess. Another factor is the greater probability, with increasing sugar concentration, of active molecules or ions condensing to higher sugars, resins, or other products and thereby being diverted from saccharinic acid formation.

It may be noted here that these two factors doubtless account for the large quantitative discrepancies between the results of Evans and coworkers and our own. Their results with hexoses

were noted above (p. 359). From the trioses Evans and Cornthwaite (16) obtained very much lower yields of lactic acid than those here reported, finding in N KOH at 50° only about 20 per cent (or 0.4 mols per 2 mols of triose compared to our 1.2 to 1.65), with maxima of 40 to 45 per cent in 4 N KOH (0.8 to 0.9 mol per 2 mols of triose, compared to our maxima of 1.6 to 1.9). They used 1 M solutions of triose, which neutralize *at the start* up to equivalent amounts of added alkali, leaving the initial alkalinity far less than corresponds to the added hydroxide. This fact was overlooked by these workers and to a large extent invalidates the relation of alkalinity to lactic acid and other products in their experiments. The fact is however of interest that 1 or even 2 equivalents of KOH (shown also in our data) are insufficient to bring about maximum conversion to lactic acid. The high sugar concentration used by Evans also directly influences the products formed, as noted above.

Influence of Temperature.

Table VI gives data on the lactic acid yield from glucose in 0.5 M KOH at different temperatures, from slightly below 0° to that of a boiling water bath. Although the destruction is much slower at low temperature, the total lactic acid ultimately formed is much greater, due presumably to a lower rate of side reactions and polymerizations leading to resins and other complex products. At low temperature the color of the solutions is less, being almost absent at 0°. An opposite effect of temperature is indicated by the recent report by Wolf (23) of 74 per cent lactic acid from cane sugar heated with 3 mols of CaO in an autoclave to 197°.

Table VII gives similar data for galactose and two pentoses, within higher temperature range. With the pentoses also the lactic acid yield decreases with rise of temperature; while with galactose the effect of temperature is slight.

Before passing to our calculations of salt fractions at the various alkalinities, we may consider briefly the facts already noted in relation to the reactions indicated in Diagram 1. It is evident that at low alkalinity (and temperature) (alkali carbonate and bicarbonate) the reactions must be largely limited to the reversible interconversions among the sugars and dienols across the center of the diagram without saccharinic acid formation or rupture.

Within this range polymerization of lower sugars to higher also occurs, with the conversion of methylglyoxal to triose and of triose to hexoses (20–23, 43). The equilibrium is toward the center and to the left of the diagram, the products converging toward aldohexoses. At the higher alkalinity of KOH (or higher

TABLE VI.

Influence of Temperature on Yield of Lactic Acid. Glucose, in 1.5 M KOH.

Temperature.	Reaction time.	Lactic acid formed.	Glucose destroyed.	Lactic acid per mol of sugar.
°C.	days	mM	mM	M
−3 to −1	215	38.7	29.7	1.30
25–26	12	55.6	48.7	1.14
25–26	24	57.0	50.0	1.14
37–38	29	54.9	50.0	1.10
60–70	1	39.4	40.0	0.98
70–95	2	41.0	50.0	0.82
68–73	3	45.7	50.0	0.91
99	3	39.9	50.0	0.80

TABLE VII.

Influence of Temperature on Yield of Lactic Acid. Sugars in 0.5 M KOH.

Sugar.	Temperature.	Reaction time.	Lactic acid formed.	Sugar destroyed.	Lactic acid per mol of sugar.
	°C.	days	mM	mM	M
d-Galactose.	37–38	29	24.9	50	0.498
	71	3	24.1	50	0.482
	99	3	24.3	50	0.487
l-Xylose.	37–38	29	30.0	60	0.500
	71	3	24.5	60	0.408
	99	3	23.7	60	0.395
l-Arabinose.	37–38	29	28.1	60	0.468
	71	3	26.2	60	0.437
	99	3	25.3	60	0.422

temperature) the enols begin to rearrange into ortho osones and saccharinic acids and to separate into smaller fragments, thus destroying the sugars. In the lower region of this range the acids are mainly from 1,2- and 2,3-dienols. With further rise of alkalinity, although the total acids formed are not much increased, the proportion as lactic acid (from trioses and the glucose series)

rises to a maximum, indicating a diversion from the other reactions, presumably because of a relative increase in the concentration of the 3,4-dienol or its salt, at the expense mainly, it seems, of the 2,3-dienol. These relations could be understood if it might be supposed that there is associated with each dienol an additional acidic group (or groups) decreasing in individual strength but increasing in their *sum* of base-binding ability, in the order 1,2-, 2,3-, and 3,4-dienol. This would require that the sugars behave as polybasic acids, which should be susceptible to test by experiment. An attempt to decide this point is now under way.

Starting with the trioses, the reactions seem somewhat more decipherable. The results show that in sufficiently dilute solutions of the sugar and at very high alkalinity almost theoretical conversion to lactic acid may take place. The same is true of methylglyoxal, even at somewhat lower alkalinity (44). In dilute alkali, however, a portion of both triose (43) and methylglyoxal (20–23) is diverted in the opposite direction, apparently to hexoses, from which a small fraction may again pass, presumably via triose and methylglyoxal, to lactic acid, the balance giving resins and other saccharinic acids. In neutral or acid solutions methylglyoxal and the trioses are stable except at high temperatures. We may picture the relations as given in the formulæ somewhat as follows: Starting at methylglyoxal (1), we suppose that it enolizes in two ways, one to give (2) which by adding water (H^+ and OH^-) gives lactic acid (3), and the other (4) which by adding water gives glyceraldehyde (5). The last is in equilibrium (?) with (*a*) 3,4-hexose dienol and (*b*) with the 1,2-triose dienol. High alkalinity evidently favors the first type of enolization, as is presumably the case also with the other ortho osones. The reverse of these changes would represent the formation of the ortho osone from the aldose. It is possible to describe this behavior somewhat acceptably in terms of electronic structure if it be permissible to regard glyoxals and dienols as dibasic acids. Justification for such views must await further work.

Relation of Lactic Acid Yield to Salt Fraction.

It would seem reasonably certain that the influence of increasing concentrations of KOH on the yield of lactic acid must be an effect

High alkalinity →
← Low alkalinity
Hexose 3,4-dienol.
Dihydroxy-acetone.
Triose 1,2-dienol.
Glyceer-aldehyde. (5)
Methylglyoxal enol. (4)
Methylglyoxal. (1)
Methylglyoxal enol. (2)
Lactic acid. (3)

of hydroxyl ions in determining the proportion of sugar present as salt or ions, possibly of second or even of third acidic groups. It was therefore of interest to attempt closer correlation of these quantities. There are unfortunately a number of difficulties which prevent satisfactory treatment. More serious than errors in the analytical results are uncertainties due to the "abnormal" behavior of strong solutions of electrolytes, such as the KOH solutions in which the formation of saccharinic acids takes place. Assumptions as to constancy of apparent hexose dissociation constants and of water activity must be made which are open to question, while the constants for the trioses are unknown. For these and other reasons the calculations here given can be regarded only as trial approximations, which nevertheless are suggestive and we believe worth recording.

We have calculated the initial hydroxyl ion *concentration* and *activity*, of the solutions as follows: concentration $p(OH)_c$, from conductance data for KOH at 18° (International Critical Tables, 6) $\left(\frac{\Lambda_c}{\Lambda_o} \times M = [OH]_c\right)$, in which M is the initial molarity of KOH, $[OH]_c$ the hydroxyl ion concentration, Λ_c and Λ_o (=235 by extrapolation) their conventional meaning). Activity $p(OH)_a$ was calculated from Scatchard's (45) activity coefficients for KOH at 25° ($\gamma_m \times M = (OH)_a$). The difference in temperature between that for which $[OH]_c$ and $(OH)_a$ are calculated and that of the lactic acid formation (37–40°) was ignored. When the molar lactic acid yields are plotted against p(OH) values thus calculated, we obtain a series of curves similar to those of Fig. 1, which give little additional information, except that in the highest alkalinity the activity data give a smoother curve than do those from conductance. In this region the activity coefficients are uncertain, though much greater than dissociation factors by equivalent conductance. (The curves are not reproduced.)

Calculation of the salt fraction of the sugars from the equation

$$pH - pK_1 = \log \frac{[A]}{[HA]}$$

requires evaluation of the pH of the solutions and pK_1 of the sugar. For calculation of pH we take the value 13.50 for pK_w at 37.5°,

and, ignoring both temperature differences and the possibility of change of pK_w in concentrated solutions, assume

13.50 − p(OH) (activity at 25° or conductance 18°) = pH (37.5°)

For pK_1 of glucose we chose the value 12.00 at 37.5° (Landolt and Börnstein give 9.8×10^{-13} at 40°). The antilog of $\left(\log \frac{[A]}{[HA]}\right) = R$, and $\frac{R}{R+1}$ = salt fraction of sugar (as the monobasic salt of the acid whose pK_1 is 12.00 at 37.5°). We thus calculate

TABLE VIII.

Calculated Salt Fraction and Lactic Acid Yield. Glucose Series.

Hexose concentration.	Initial KOH concentration.	$(OH)_a$ activity.	$(OH)_a$ corrected activity.	pH (37.5°).	pH corrected (37.5°).	Salt fraction pK_1 = 12.00.	Salt fraction corrected.	Lactic acid yield.		
								Per mol hexose.	Fraction of maximum.	Fraction of theory (2 mols).
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
mM	*M*	*M*	*M*					*M*		
5	0.02	0.0174	0.013	11.74	11.61	0.36	0.29	0.55	0.47	0.27
5	0.04	0.0334	0.029	12.02	11.96	0.51	0.48	0.71	0.61	0.35
10	0.10	0.0772	0.069	12.39	12.34	0.71	0.69	0.85	0.72	0.42
10	0.25	0.183	0.176	12.76	12.75	0.85	0.85	1.04	0.89	0.52
50	0.50	0.361	0.325	13.06	13.01	0.92	0.91	1.06	0.91	0.53
50	1.00	0.743	0.706	13.37	13.35	0.96	0.96	1.15	0.98	0.57
50	2.00	1.70	1.66	13.73	13.72	0.98	0.98	1.17	1.0	0.58
50	5.00	(7.50)	(7.42)	(14.38)	(14.37)	(0.99)	(0.99)	1.08	0.92	0.54
17.5	10.00	(20.00)	(20.00)	(14.80)	(14.80)	(1.00)	(1.00)	1.10	0.94	0.55

Figures in parentheses are less reliable values obtained by extrapolation.

the data given in Table VIII. Only the data calculated from activity coefficients are given, since these appear to be more consistent in the stronger solutions. In the lower ranges there is no significant difference between results calculated by the two methods.

In Column 5 the pH and in Column 7 (Table VIII) the salt fraction is calculated without taking account of base bound by sugar or by acids formed, while in Columns 6 and 8 the same values are calculated on the assumption that the added sugar neutralized and removed 1 equivalent of base. The point of

interest is the comparison of the calculated salt fractions with lactic acid yield. In the two lower concentrations of KOH (0.02 and 0.04 M) the lactic acid yield (in fraction of maximum) exceeds the salt fraction, and in the two strongest solutions a smaller discrepancy appears in the opposite direction. Otherwise the agreement is good. It does not seem probable that the fair agreement is fortuitous. We are disposed to interpret the relation to mean that the *final amount* of lactic acid formed from these sugars is within the limits given *proportional to the fraction of the sugar*

TABLE IX.

Calculated Salt Fraction for Assumed Dissociation Constants. Dihydroxyacetone, 10 mM.

Initial KOH.	pH_a corrected.	Salt fraction $pK_1 = 12.00$.	Salt fraction $pK_1 = 12.80$.	Lactic acid yield per mol triose.	Lactic acid / Salt fraction	
					pK 12.00	pK 12.80
(1)	(2)	(3)	(4)	(5)	(6)	(7)
M				M		
0.02	11.44	0.22	0.03	0.25	1.14	8.3
0.04	11.90	0.44	0.11	0.32	0.73	3.0
0.10	12.34	0.69	0.26	0.40	0.58	1.5
0.25	12.75	0.85	0.47	0.53	0.62	1.12
0.50	13.05	0.92	0.64	0.67	0.73	1.05
1.0	13.37	0.96	0.79	0.82	0.85	1.04
2.0	13.75	0.98	0.90	0.90	0.92	1.0
5.0	(14.37)	0.99	0.97	0.93	0.94	0.96
10.0	(14.80)	1.0	0.99	0.95	0.95	0.96

Figures in parentheses are less reliable values obtained by extrapolation.

present as salt or ions. The results indicate that the ion is that of the *primary* acidic group, which is contrary to our expectation. (The *rate* of formation shows a different relation.) We have no explanation why the lactic acid formed is higher than expected at low pH, unless it be that the lactic acid results in this range are erroneously high due to presence of much 1,3-dihydroxybutyric acid. Another point should be noted: the dissociation constants of fructose and mannose are higher than that of glucose (9), and if the above conclusion be valid, it might be expected that they would yield somewhat more lactic acid at a given (lower) pH than glucose. The results give no evidence of such differences in yield. It may be thought that at such high alkalinity each of

these sugars is converted into substantially the same mixture, though in a later paper it will be shown that the *rates* of acid formation are different for each sugar.

We have attempted to calculate the salt fractions similarly for the trioses. Here the calculation becomes more venturesome, since besides the difficulties already noted, the dissociation constants of the sugars are unknown. We may however try various hypothetical values for pK_1 and infer that the one yielding data corresponding to lactic acid may approach the correct one. Table IX gives data for the salt fraction calculated for values of pK_1 of 12.00 and 12.8 for dihydroxyacetone (10 mM as to triose). The values of pH and salt fraction are calculated on the assumption that the KOH concentration is reduced by 1 equivalent of the triose added. Otherwise the calculations are based on the same assumptions as with the glucose series. Except in 0.02 M KOH the results are not greatly different when no correction is made for base bound by sugar. On comparing the calculated salt fractions with the yield of lactic acid expressed as fraction of the maximum theoretical value (1 mol per mol of triose), we find *only* at the lowest alkalinity (0.02 M KOH) good agreement for a pK_1 value of 12.00, the same used for hexose; at higher alkalinity the lactic acid yield falls far short of the calculated salt fraction. But with increasing alkalinity there is an approach to good agreement with the salt fractions calculated for a pK value of 12.8 (Columns 4, 5, and 7). This would seem to suggest that the *direct* conversion of dihydroxyacetone into lactic acid under the influence of alkali is proportional to the fraction of the sugar present as salt (ions), the sugar having an apparent acidic dissociation constant (perhaps the second) of about 1.6×10^{-13}.

In the lower range of alkalinity where this relation does not hold there is reason to believe that the deviation is due to partial conversion of the triose to hexose. This conversion is known to occur at low alkalinity (Fischer and Tafel (43)). Furthermore comparison of the yields of lactic acid from triose (2 mols) with that from the glucose series of hexoses at the same alkalinity shows them to be very similar in the low range of alkalinity 0.02 to 0.10 M KOH at which the above deviation occurs.

It should be noted that since saccharinic acid formation is a slow process it might be expected that alkalinity would affect the

rate, but it is not equally evident why alkalinity should influence *qualitatively* the acids formed. It is this *qualitative* difference which is involved in the relation considered above. The only explanation of this influence appears to be that the degree of alkalinity determines the proportions of several different salts (or different dienols). In another paper somewhat similar though quantitatively different relations between *rates* of reaction and alkalinity will be discussed.

SUMMARY AND CONCLUSIONS.

Data are reported on the amounts of total saccharinic acids and of lactic acid formed from hexoses, pentoses, and trioses under the influence of varying concentrations of KOH. The influences of hydroxyl ion concentration, sugar concentration, and temperature are considered. High alkalinity favors, while high temperature and high sugar concentration lower the yield of lactic acid. Within limits the yield of lactic acid from glucose appears to be a function of the fraction of the sugar present in the form of salt. At quite high alkalinity and low sugar concentration dihydroxyacetone is almost quantitatively converted to lactic acid, the yield being apparently a function of a salt fraction of the triose, calculated for an assumed pK_1 value of 12.80. It is concluded that alkalinity and temperature "activate" the sugars by salt formation and consequent ionization, and that saccharinic acid formation may be regarded as the spontaneous rearrangement of unstable sugar ions. The ratio of particular acids formed probably depends upon the proportions of *different* salts or ions.

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THE MECHANISM OF EPINEPHRINE ACTION.

V. CHANGES IN LIVER GLYCOGEN AND BLOOD LACTIC ACID AFTER INJECTION OF EPINEPHRINE AND INSULIN.

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Carbohydrate balances were made in rats after injection of epinephrine under three different conditions; in fasting rats, in rats in the postabsorptive state, and in rats during glucose absorption (1–3). In the first and second case an increase in liver glycogen was observed 3 hours after the injection. In the last case attention was paid to the time relations by determining the liver glycogen after 1, 2, 3, and 4 hours of glucose absorption. When epinephrine was injected the liver glycogen was higher after 2, 3, and 4 hours, while after 1 hour there was no difference between control and injected animals.

That epinephrine may cause an increase in liver glycogen was already known in the case of fasting rabbits. Experiments under other than fasting conditions had not been attempted, owing, no doubt, to the extreme individual variability of the liver glycogen of well fed rabbits. The time intervals in the experiments on rabbits were much longer than in those on rats; in the former animals the increase in liver glycogen was demonstrated 7 to 24 hours after the epinephrine injection, while in rats the increase had completely disappeared after 16 hours. This made it appear probable that, though the same metabolic changes occurred in both species, the rate at which they took place was much faster in rats, which is easily understood if one considers the high metabolism of this species as compared to rabbits.

The increase in liver glycogen was formerly regarded as an irregular occurrence, not really characteristic for epinephrine and limited to the fasting condition. It was also regarded as proof of a conversion of fat into carbohydrate. The experiments in

which a complete carbohydrate balance was made revealed the material from which the liver formed the excess glycogen and it became evident that new formation of liver glycogen is one of the characteristic effects of epinephrine. This hormone through its action on muscle glycogen raises the blood lactic acid level and the lactic acid is used by the liver in the formation of glycogen. But epinephrine leads not only to new formation but also to mobilization of liver glycogen and it depends therefore on the relative rate of the two processes whether the liver glycogen decreases, increases, or remains unchanged.

Each of the three possibilities mentioned can be demonstrated experimentally. The increase in liver glycogen has already been discussed. Observations concerning a decrease in liver glycogen after epinephrine injections in other species than rats were recorded by many investigators and their work was reviewed in a previous paper (2). A decrease was observed in this laboratory when successive pieces of liver were removed from rabbits under amytal anesthesia (4). However, the demonstration of a fall in liver glycogen after epinephrine injections has lost much of its former importance since it has been shown definitely on hepatectomized animals that liver glycogen is the only direct source of blood sugar in the body. This makes it clear that the excess of sugar in the blood after epinephrine injections comes from liver glycogen, irrespective of whether the latter decreases or increases after the injection.

The present experiments were undertaken in order to ascertain whether or not a decrease in liver glycogen could also be demonstrated in the rat. Previous observations made it seem probable that such a decrease would occur at time intervals shorter than 1 hour. Determinations of liver glycogen made very soon after the epinephrine injections have the advantage that at that time the synthesis from lactic acid has not yet started, so that a decrease in liver glycogen may be regarded as a true index of the rate of hepatic glycogenolysis.

EXPERIMENTAL.

For a study of glycogenolysis it was desirable to use animals with high glycogen stores. Well fed rats taken directly from their cages show a marked individual variability of liver glycogen and

are therefore unsuitable. To insure a uniform and high content of liver glycogen in the different animals the following procedure was adopted. Male and female rats of 120 to 160 gm. of body weight were used, all bred in this laboratory and fed and kept under uniform conditions. The animals were fasted 24 hours to reduce the liver glycogen to a very low and uniform level. They were then fed 600 mg. of glucose per 100 gm. of body weight, the sugar solution being introduced by stomach tube. From previous experiments (3) it is known that absorption of this amount is complete in 3 hours. To insure a further margin of safety, the injections were made 4 hours after the glucose feeding. The same dose of epinephrine as in previous work was used, namely 0.02 mg. per 100 gm. of body weight, injected subcutaneously. The controls were given 0.15 cc. of salt solution subcutaneously. When it was found that epinephrine diminished the glycogen in the first 15 minutes, it seemed of interest to study the effect of insulin on this glycogenolysis. Experiments with insulin alone had also to be included and in both cases 0.24 units per 100 gm. of body weight were injected. The rats were killed by decapitation ¼, 1, and 3 hours after the injections and 1 to 2 cc. of blood were collected. The liver was rapidly excised, frozen in a stream of CO_2 delivered from a tank, weighed in the frozen state, and introduced into boiling 60 per cent KOH. Glycogen was determined according to Pflueger's method. NaOH was added to the acid hydrolysate of the glycogen until the reaction was only slightly acid to litmus, impurities were removed by a short agitation with Lloyd's reagent, and the solution was filtered. Suitable control experiments showed that this procedure neither removes sugar nor adds any reducing substance to the filtrate. The sugar in the glycogen hydrolysate was determined by means of Bertrand's method. Blood sugar was determined according to the Hagedorn and Jensen (5) and lactic acid according to the Friedemann and Kendall (6) method in the same Folin-Wu filtrate.

Table I summarizes all experiments performed. The average error (of a single result) was calculated in order to illustrate the extent of individual variation. Owing to the fact that both sexes were used and that the experiments were carried out over a long period of time, a somewhat larger variability in liver glycogen content was encountered than in previous work, but this is offset by

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TABLE I.

Effect of Epinephrine and Insulin on Liver Glycogen, Blood Sugar, and Blood Lactic Acid of Well Fed Rats.

0.02 mg. of epinephrine and 0.24 units of insulin were injected per 100 gm. of body weight.

Controls.					Injected with epinephrine.					Injected with insulin.					Injected with insulin plus epinephrine.				
Per 100 gm. rat.		Liver glycogen.	Blood sugar.	Blood lactic acid.	Per 100 gm. rat.		Liver glycogen.	Blood sugar.	Blood lactic acid.	Per 100 gm. rat.		Liver glycogen.	Blood sugar.	Blood lactic acid.	Per 100 gm. rat.		Liver glycogen.	Blood sugar.	Blood lactic acid.
Weight of liver.	Glycogen in liver.				Weight of liver.	Glycogen in liver.				Weight of liver.	Glycogen in liver.				Weight of liver.	Glycogen in liver.			
15 min. after injection.																			
gm.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*gm.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*gm.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*gm.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*
3.20	77.8	2.43	115	10.7	3.18	48.4	1.52	171	30.2	3.25	95.3	2.93	131	15.7	3.10	50.5	1.62	117	20.4
3.44	72.4	2.10	127	19.1	3.22	44.0	1.37	178	27.1	3.16	57.4	1.81		12.9	3.30	104.1	3.15	173	26.7
3.23	74.5	2.31	121	19.0	3.15	52.0	1.64	150	27.0	3.39	76.6	2.26	126	23.2	3.21	81.1	2.55	115	32.4
3.13	73.4	2.34	126	18.5	3.13	82.2	2.63	159	33.7	3.30	92.9	2.80	116	18.2	3.61	95.6	2.65		34.4
3.27	86.4	2.64	138	12.1	3.12	59.5	1.90	160	36.5	3.20	81.3	2.54	151	22.4	3.37	70.8	2.10	175	27.7
3.51	66.3	1.89		19.9	3.30	38.6	1.17	204	34.6	4.00	66.4	1.65	124	20.5	3.60	71.2	2.00	123	23.6
4.18	89.8	2.15	137	14.3	3.52	56.9	1.61	149	23.3						3.52	49.4	1.40	113	31.0
3.54	128.7	3.64	138	13.7															
3.44	83.7 ±13	2.44	129	15.9	3.23	54.5 ±10	1.69	167	30.3	3.38	78.3 ±11	2.33	129	18.8	3.39	74.7 ±16	2.21	136	28.0

1 hr. after injection.																		
3.56	72.1	2.02	120	9.6	3.51	83 0	2.36	227	35.2	2.52	20.4	0.81	84	17.5	2.93	13.0	0.44	105	22.1
3.10	107.1	3.46	135	13.6	3.03	88.0	2.90	222	29.8	2.99	9.9	0.33	87	19.2	2.76	26.2	0.45	122	36.0
2.92	37.8	1.30	122	11.1	3.71	79.2	2.12	252	25.7	3.43	80.0	2.33	87	26.2	3.20	4.8	0.15	72	27.2
3.14	89.4	2.84	141	9.7	3.16	37.5	1.18	189	26.6	2.87	16.0	0.55	77	19.6	3.41	5.7	0.17	80	13.2
3.54	74.8	2.11	135	11.4	3.96	73.7	1.86	243	39.0	3.56	51.5	1.44	80	15.0	3.63	7.3	0.20	80	28.4
					4.03	97.7	2.42	222	33.4	3.67	38.2	1.04	67	18.7	3.26	39.0	1.20	116	35.5
															3.22	33.0	1.02	121	32.4
3.25	76.2 ±18	2.35	131	11.1	3.57	76.5 ±14	2.14	226	31.6	3.17	36.0 ±21	1.08	80	19.4	3.20	18.4 ±12	0.52	99	27.8
3 hrs. after injection.																			
3.67	81.2	2.21	132	14.0	3.51	109.5	3.12	155	8.1										
3.83	102.2	2.67	127	16.2	3.78	170.1	4.48	163	9.7										
3.42	78.6	2.29	124	7.0	3.25	89.1	2.74	161	11.9										
2.38	49.9	2.10	111	11.9	3.14	80.7	2.84	143	14.1										
3.56	23.8	0.67	124	15.7	4.08	158.1	3.85	149	5.9										
3.46	89.3	2 57	126	6.2															
3.39	70.8 ±23	2.08	124	11.8	3.55	121.5 ±34	3.41	154	9.9										

the number of observations made. Also, on calculating the average error of the mean it is found that the changes brought about by the injection of epinephrine and insulin are significant.

The averages of the three control groups in Table I show that there occurs a very slow decrease in liver glycogen in the first few hours of fasting. While the liver contained an average of 83.7 mg. of glycogen 1 hour and 15 minutes after the ceasing of glucose absorption, this value fell to 70.8 mg. in the next 3 hours. An injection of epinephrine under these conditions is followed by a drop in liver glycogen in 15 minutes which is larger than the decrease observed during a period of 3 hours without injection. After 1 hour the injected rats contain the same amount of liver glycogen as the control rats, which means that the loss of glycogen sustained in the first 15 minutes is made good by new formation of liver glycogen. 3 hours after the epinephrine injection this new formation of liver glycogen has resulted in a marked increase in liver glycogen, the average difference being 50.7 mg. in favor of the injected rats. (In previous experiments under similar conditions and for the same time interval the rats injected with epinephrine contained on an average 73 mg. more liver glycogen than the control animals (2).)

The correlation between new formation of liver glycogen and blood lactic acid is of interest. It will be noted in Table I that 15 minutes after the epinephrine injection the blood lactic acid has already risen. 1 hour after the injection the blood lactic acid is practically the same as after 15 minutes and after 3 hours the blood lactic acid has returned to its normal level. The period of increase in liver glycogen is therefore coincident with the period of decrease in blood lactic acid. The blood sugar which reaches its peak value 1 hour after the injection is still markedly elevated 3 hours after the epinephrine injection. Glycosuria did not occur in these experiments.

The experiments in Table I in which insulin alone was injected show no change in liver glycogen and blood sugar after 15 minutes and a decrease in both after 1 hour. Though insulin apparently produces no effect in the first 15 minutes after the injection, it checks the glycogenolytic action of epinephrine occurring during that time. This is shown by the fact that 15 minutes after the simultaneous injection of both insulin and epinephrine the average

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liver glycogen and blood sugar is very close to that of the control animals, while the injection of epinephrine alone leads to a decrease in liver glycogen and an increase in blood sugar. 1 hour after the injection of both hormones, insulin action is in the preponderance since the effect is similar to that produced by the injection of insulin alone; *i.e.*, both liver glycogen and blood sugar are diminished. Insulin suppresses the epinephrine hyperglycemia in 60 per cent of the cases after 15 minutes and in all cases 1 hour after the injection of both hormones. Insulin, however, has very little effect on the increase in blood lactic acid resulting from the epinephrine injection.

DISCUSSION OF RESULTS.

By choosing a very short time interval after the epinephrine injection (15 minutes), a decrease in liver glycogen has been demonstrated in the rat. Though it could be inferred in previous experiments that liver glycogen was being mobilized, an actual decrease was not encountered when analyses were made 1, 2, 3, and 4 hours after the injection. As is apparent now, the demonstration of a fall in liver glycogen in the rat is possible only shortly after the injection because at that time glycogenolysis is rapid while the opposing process of glycogen synthesis has not attained its full rate. It requires a few minutes until the lactic acid and sugar concentration in the blood have risen sufficiently high to set glycogen synthesis in motion. As soon as this synthesis of liver glycogen from lactic acid (and blood sugar) starts, the fall in liver glycogen is not only arrested but an actual gain in liver glycogen is found some hours after the injection. The previously negative glycogen balance in the liver becomes positive and in this way the simultaneous occurrence of mobilization of liver glycogen is obscured.

The foregoing analysis is in harmony with the concept developed in a previous paper (2) that the glycogen content of the liver always depends on the relative rate of glycogen synthesis and glycogenolysis. The divergent results recorded in the literature as to the behavior of liver glycogen after epinephrine injections were explained on that basis and it was pointed out that a marked decrease in liver glycogen was to be expected mainly in the case of glycosuria. To this may be added the observation that the

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combination of anesthesia and epinephrine injection results in a low liver glycogen because the anesthetic retards or inhibits synthesis of lactic acid to liver glycogen and enhances glycogen mobilization (7).

What remained unknown in previous experiments was not the fact that liver glycogen may diminish under certain conditions but the actual rate of either glycogen synthesis or glycogenolysis. The present experiments give some insight into the rate of the latter process, because it might be assumed that in the first 15 minutes of epinephrine action only one process occurs or, at any rate, that mobilization of liver glycogen is greatly in preponderance.

According to Table I 29 mg. of liver glycogen disappeared in 15 minutes, or, if the same rate of mobilization were to prevail in later periods of epinephrine action, 116 mg. would be mobilized per 100 gm. of body weight per hour. This is by no means a large amount of sugar for the rat, because twice as much sugar is utilized per hour after a carbohydrate meal (8). But even so, there is good reason for believing that the rate of mobilization of liver glycogen diminishes in later periods of epinephrine action. This is based on the following evidence. According to Table I the glycogen content of the liver 1 hour after the epinephrine injection is higher than after 15 minutes. This increase must be due to the fact that between 15 minutes and 1 hour glycogen synthesis proceeds faster than glycogenolysis. To the latter we assigned a rate of 116 mg. per hour under the assumption that the initial rate is maintained. In this case, as may easily be calculated, glycogen synthesis must proceed at a rate of 145 mg. per hour in order to account for the glycogen content of the liver found 1 hour after the injection. Such a high rate of glycogen formation in the liver from lactic acid has not been observed in previous experiments; in fact, the maximum amount of glycogen deposited during *d*-lactic acid absorption from the alimentary canal was only 20 mg. per hour (9) or 7 times less than the amount required. It is quite likely that blood sugar is reconverted into liver glycogen during epinephrine action, but even glucose has not yielded a higher rate of glycogen formation in the liver than 60 mg. per hour (3) which together with the 20 mg. of glycogen derived from lactic acid would still fall short of the required 145 mg. The discrepancy is still larger when the same calculation is made for the

3 hour period of Table I, since the injected animals show a gain of 45 mg. of liver glycogen between the 1 and 3 hour periods.

The facts just presented indicate that glycogen mobilization in the liver cannot continue at its initial rate, because glycogen synthesis cannot proceed at the required rate of 145 mg. per hour. If one assigns to formation of liver glycogen from glucose plus lactic acid the maximal observed rate of 80 mg. per hour, glycogenolysis must be reduced accordingly in order to account for the increase in liver glycogen of 22 mg. which was observed between 15 and 60 minutes (Table I). A calculation made on this basis gives the values recorded in Table II.

According to Table II the rate of mobilization of liver glycogen which was 116 mg. per hour in the first 15 minutes, dropped to 51

TABLE II.

Calculated Rates of Synthesis and Mobilization of Liver Glycogen after Epinephrine Injection.

Liver glycogen.	0 to 15 min.	Rate per 100 gm. of body weight of rat per hr.	15 to 60 min.	Rate per 100 gm. of body weight of rat per hr.
	mg.	*mg.*	*mg.*	*mg.*
Synthesis	0	0	+60	80
Mobilization	−29	116	−38	51
Balance	−29		+22	

mg. per hour in the next 45 minutes of epinephrine action. It is assumed that from then on the rate of mobilization diminishes still further because the blood sugar and lactic acid concentration begin to fall. This reduces the rate of synthesis and consequently the rate of mobilization must also be reduced. One finds therefore a constantly diminishing rate of hepatic glycogenolysis after subcutaneous injection of epinephrine.

There are several factors which could account for a decrease in the rate of glycogen mobilization after the first 15 minutes. One possibility is that glycogen synthesis which sets in has a retarding effect on glycogenolysis or that glycogenolysis is a self-inhibitory process. Both cases amount to much the same thing, if one depicts glycogen synthesis and glycogenolysis in the liver as an equilibrium between glucose $\rightleftarrows$ glycogen. In favor of this

assumption is the fact that the rate of glycogen formation in the liver was found to depend on the height of the blood sugar level and hence on the height of the glucose concentration in the liver (7). It should also be mentioned that during the conversion of sodium lactate into liver glycogen base is liberated which might retard the activity of the diastatic enzyme. Sahyun and Luck (10) have offered another explanation. They believe that epinephrine may temporarily be stored in the liver and then released again and that this accounts for the hepatic phase of epinephrine action; *i.e.*, for the phase during which hydrolysis of hepatic glycogen is accelerated. They cite as evidence their finding that injected amino acids are temporarily stored in the liver. However, epinephrine is not a metabolite, it is present only in minutest traces in the blood and it is destroyed extremely rapidly in blood and tissues, objections which the authors themselves appreciate. Furthermore, Sundberg (11) and Markowitz and Mann (12) have shown quite conclusively that the liver is particularly active in the destruction of epinephrine in comparison to other organs.

Though the idea of a temporary accumulation of epinephrine in the liver has little in its favor, the authors have used it to supplement the theory of the writers in regard to the mechanism of epinephrine action. Sahyun and Luck found that there was a decrease in liver glycogen of rabbits 1, 1½, and 2 hours after the epinephrine injection. After 3 hours the hepatic glycogen had risen beyond the original value and thenceforth it continued to rise up to the 18th hour, by which time a considerable deposition had occurred. Taking into consideration the fact that changes in glycogen distribution are much more rapid in the rat than in the rabbit on account of the higher metabolism of the former species, the observations by Cori and Cori recorded in this and in previous papers and those of Sahyun and Luck are in excellent agreement.[1] The latter authors are also of the opinion that lactic acid derived from muscle glycogen is the source for the increase in liver glycogen and that a decreased utilization of blood sugar is an important factor in epinephrine hyperglycemia.

[1] Sahyun and Luck, by paying attention to the time relations, have brought the new fact to light that the sequence of events is first a decrease and then an increase in liver glycogen. This result they kindly communicated to us while their paper was in press.

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In view of this it is perhaps of minor importance that Sahyun and Luck imply, erroneously as we believe, that Cori and Cori have not considered the possibility of a decrease in liver glycogen as the result of increased glycogenolysis and that their theory needs to be supplemented in this respect. The last named authors stated on the basis of a review of the literature that (13) ". . . . it is left to future work to discover under what conditions and after what doses of epinephrine liver glycogen diminishes and especially how much liver glycogen disappears." These conditions have now been accurately determined for the rabbit by Sahyun and Luck and for the rat by the present authors.[2]

A correlation to the organism as a whole is necessary in an attempt to evaluate the significance of the decrease in liver glycogen after epinephrine injections. The liver of rabbits constitutes about 4 and the muscles 50 per cent of the body weight and it can therefore be seen that recording changes in these organs in terms of glycogen percentage must give a distorted picture. The percentage changes in the liver will appear relatively large in comparison to those in muscle and hence one is easily misled into overemphasizing the changes in the former. The picture is entirely different when the amounts of glycogen which disappear from liver and muscles of the whole animal are calculated. In Table III the experiments of Sahyun and Luck have been recalculated in this manner, Tables II and III of their paper, where glycogen values are expressed in per cent, being used.

The results shown in Table III are very striking. In the first hour, in which according to the analysis of Sahyun and Luck the hepatic action of epinephrine is very pronounced, 17 times as much glycogen disappears from the muscles as from the liver. This bears out in a most convincing manner the contention made by the present writers that (2) ". . . . the most prominent effect of epinephrine is observed in the peripheral tissues."

In previous experiments it was shown that the amount of sugar offered to the body by the liver during 3 hours of epinephrine action was small, when compared with the amount of sugar that a rat utilizes after a carbohydrate meal. However, an exact

[2] There is this difference, however, that Sahyun and Luck injected glycosuric doses of epinephrine, while in the corresponding experiments on rats glycosuria was not produced.

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estimate of the amount offered could not be made because after 3 hours new formation of liver glycogen overbalanced glycogen mobilization. The present paper, which includes a reasonable estimate of the rate of glycogenolysis in the liver of the rat at different time intervals after the epinephrine injection, supplements previous experiments in this respect. The data of Tables I and II show that epinephrine undoubtedly accelerates the glycogenolytic process. But even in the 1st hour of epinephrine action the amount of sugar entering the blood from the liver is by no means large (about one-fourth of what a rat can use per hour) and

TABLE III.

Changes in Liver and Muscle Glycogen of Rabbits Weighing 2 Kilos after Epinephrine Injections.

Calculations were made from the data of Sahyun and Luck (10). Liver weight was assumed to be 3.7 and muscle weight 47 per cent of body weight (14).

	Glycogen in liver.	Change in liver glycogen.	Glycogen in muscles.	Change in muscle glycogen.	No. of experiments.
	mg.	*mg.*	*mg.*	*mg.*	
Before injection (controls)....	313		4680		4
After injection.					
1 hr.....	74	−239	526	−4150	2
1½ hrs.....	55	−258	338	−4340	3
2 "	195	−118	698	−3980	4
3 "	512	+199	435	−4240	4
4 "	592	+279	526	−4150	4
5 "	970	+657	279	−4400	4

cannot explain the resulting hyperglycemia without a simultaneous decrease in the rate of blood sugar utilization. In this our conception differs radically from that of previous workers who explained the hyperglycemia solely by an excess of sugar coming from the liver (*i.e.*, more than the animal can use) and disregarded the extrahepatic action of epinephrine. Since an increased rate of hepatic glycogenolysis was already recognized by us as a part of epinephrine action, there seems to be no need for the proposal of Sahyun and Luck to combine our theory and the overproduction theory. These authors are right, however, in their contention that the rate of hepatic glycogenolysis after subcutaneous injection

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is higher in the beginning than in subsequent periods and in view of this it would be desirable to know whether the same is true when epinephrine is injected intravenously at a constant rate.

Injection of both insulin and epinephrine resulted in an almost complete suppression of the decrease in liver glycogen and increase in blood sugar observable 15 minutes after the injection of epinephrine alone. Insulin, however, did not suppress the rise in blood lactic acid regularly occurring after epinephrine injections in rats, an observation which had been made previously on rabbits (15). 1 hour after the injection of both hormones the insulin effect was predominant and observations 3 hours after the injection seemed therefore of little interest. Sahyun and Luck found in rabbits that insulin prevents the decrease in liver glycogen otherwise occurring 1½ hours after the epinephrine injection. Considering again the time relations, one sees that the results obtained on rabbits and on rats are in complete agreement and the explanation advanced that insulin inhibits the glycogenolytic process in the liver which is accelerated by epinephrine, seems by far the most probable. No contradiction is seen in the fact that insulin alone causes a decrease in liver glycogen in later periods of its action, because this decrease is always associated with hypoglycemia which in itself may lead to active hydrolysis of liver glycogen.

SUMMARY.

1. Blood sugar, blood lactic acid, and liver glycogen were determined in well fed rats at various time intervals after subcutaneous injection of epinephrine, insulin, or both hormones.

2. 15 minutes after the epinephrine injection liver glycogen was lower, after 1 hour it was the same, and after 3 hours it was higher than in control animals. Blood lactic acid which was augmented after 15 minutes and after 1 hour, had returned to normal 3 hours after the injection, while blood sugar was still elevated at that time.

3. On the basis of the available data it was calculated that the sugar set free in the liver in the 1st hour of epinephrine action amounted to only one-fourth of what a rat can use per hour. This confirms the previous contention that decreased utilization of blood sugar plays an important rôle in epinephrine hyperglycemia.

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4. 15 minutes after the insulin injection liver glycogen and blood sugar were unchanged, while after 1 hour both had diminished. When insulin and epinephrine were injected, the former prevented the decrease in liver glycogen and the increase in blood sugar regularly observed after the injection of the latter. Insulin is therefore an inhibitor of the hepatic glycogenolysis accelerated by epinephrine. Insulin has, however, very little effect on the increase in blood lactic acid resulting from the injection of epinephrine.

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A METHOD OF SEPARATION OF RIBOPOLYNUCLEOTIDES FROM THYMONUCLEIC ACID AND ON THE CONDITIONS FOR A QUANTITATIVE SEPARATION OF THE PURINE BASES FROM THE RIBOPOLYNUCLEOTIDES.

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Until the publication in 1919 of the paper by Feulgen,[1] it had been generally accepted that in animal tissues ribonucleotides occurred only in the form of mononucleotides. In that publication a new view was presented on the origin of the guanylic acid isolated from the pancreas gland. Feulgen found that in this gland guanylic acid was present as a part of polynucleotides, namely as a mixed polynucleotide, in which one nucleotide was of the ribonucleotide type and the remaining part was the usual thymonucleic acid type. A similar claim was made independently and simultaneously by E. Hammarsten.[2] However, somewhat later this author,[3] in cooperation with Jorpes, came to the conclusion that the polynucleotide, giving rise to guanylic acid, was composed entirely of ribonucleotides from which the guanylic acid could be removed leaving a polynucleotide containing only one purine component, namely adenine. The authors at that time regarded their work as incomplete and their conclusions as tentative. Finally, Jorpes,[4] continuing the work, came to the conclusion that the polynucleotide giving rise to guanylic acid was a pentanucleotide of the ribonucleotide type.

* Fellow of The Rockefeller Foundation.

[1] Feulgen, R., *Z. physiol. Chem.*, **108**, 147 (1919–20).

[2] Hammarsten, E., *J. Biol. Chem.*, **43**, 243 (1920).

[3] Hammarsten, E., and Jorpes, E., *Z. physiol. Chem.*, **118**, 224 (1922).

[4] Jorpes, E., *Acta med. Scand.*, **68**, 503 (1928).

In the course of his work, Jorpes realized the unsatisfactory state of the methods of separation of the ribopolynucleotide from the thymopolynucleotide and he also realized the unsatisfactory state of the methods of the quantitative isolation of the two purine bases. Thus, it seemed desirable to develop more satisfactory methods for these two objectives. Such methods are described in the present publication.

The method of separation of the two polynucleotides is based on the difference in the precipitability of the two substances by glacial acetic acid. The ribonucleic acid is readily precipitated from a 10 per cent aqueous solution by an excess of glacial acetic acid, whereas the thymonucleic acid under similar conditions is not precipitated at all.

For the isolation of the purine bases from the ribonucleic acid the method recommended by Levene[5] for thymonucleic acid had to be modified in its details, for the reason that the ribonucleic acid is more resistant towards the action of hydrogen chloride. A suspension of ribonucleic acid in dry methyl alcohol is not acted upon unless a definite volume of alcohol is used and unless the hydrogen chloride is passed for a considerable time. When 5 gm. of the acid are suspended in 150 cc. of the dry methanol and the gas is passed through the suspension and the suspension is agitated continuously during the operation, the nucleic acid is completely dissolved in the first phase of the operation, later the hydrochlorides of the bases separate out very gradually. The entire operation is continued for $1\frac{1}{2}$ hours and the reaction mixture is allowed to stand in the cold overnight.

The separation of the two bases is accomplished by dissolving the hydrochlorides in an excess of alkali and neutralizing the solution with acetic acid; guanine settles out immediately, adenine remaining in solution. For complete separation it is necessary to redissolve the guanine fraction in alkali and reprecipitate it with acetic acid. Adenine is precipitated from the combined filtrates as a picrate. When pure guanine hydrochloride was treated in this manner the base was recovered without loss, and from a known mixture of the two bases the recovery was accomplished satisfactorily. For comparison the bases were estimated also by the silver method.

[5] Levene, P. A., *J. Biol. Chem.*, **48**, 177 (1921).

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This method was applied to a sample of yeast nucleic acid and the bases were obtained in theoretical yields. The method was then applied to two samples of the pancreas ribopolynucleotides, prepared by Jorpes in the Stockholm laboratory and purified in

TABLE I.

		Guanine.	Adenine.	Molar ratio, guanine:adenine.	Total amount of purine bases.	N in purine bases.	N in pyrimidine bases.	Phosphoric acid hydrolyzed in 2½ hrs. with 5 per cent H_2SO_4.
		per cent	*per cent*		*per cent*	*per cent of total N*	*per cent of total N*	*per cent of total P*
Found.								
Yeast nucleic acid.								
Hydrolysis with H_2SO_4.	I.	12.5	9.2	1.2:1	21.7	65.7		
	II.	12.5	13.7 (?)				31.2	54.5
Hydrolysis with HCl in dry methyl alcohol.	I.	13.5	10.1	1.2:1	23.6		27.2	
	II.	12.3	10.2	1.1:1	22.5		32.0	
Pancreas nucleic acid.								
Hydrolysis with H_2SO_4.	I.	19.7	6.0	2.9:1	25.7	74.7		65 5
	II.	18.7	11.6 (?)				25.9	63 0
Hydrolysis with HCl in dry methyl alcohol.	I.	19.7	5.4	3.3:1	25.1		22.2	
	II.	23.1	4.5	4.6:1	27.6		24.0	
Calculated.								
A tetranucleotide (yeast nucleic acid).		11.6	10.4	1:1	22.0	66.7	33.3	53
A pentanucleotide containing 2 mols guanylic acid.		18.3	8.2	2:1	26.5	75	25	63
A hexanucleotide containing 3 mols guanylic acid.		22.7	6.8	3:1	29.5	80	20	69

this laboratory by the glacial acetic acid process. The results obtained on these samples differed greatly from those obtained on the yeast nucleic acid. The proportion of guanine to adenine was not 1 : 1 as in yeast nucleic acid but 3.3 : 1 in one sample and 4.6 : 1 in the other. The sum of the two bases is also higher

than in the yeast nucleic acid but this latter difference is not as striking as the difference in the proportions (Table I). The possibility therefore has to be considered that the guanine fraction may consist in part of oxyadenine, particularly in view of the fact that Buell and Perkins[6] have isolated oxyadenine from pig blood. Incidentally, it may be remarked that the pentose content in nucleic acids of the two types differed considerably, being higher in the pancreas polynucleotide. This observation is in harmony with the one made earlier by Jorpes,[4] which in addition to other reasons, caused him to formulate the structure of the pancreas ribonucleic acid as a pentanucleotide. The details of the structure of this nucleic acid, however, are not the subject of this communication save to show that this substance has the general character of ribonucleic acids inasmuch as on partial hydrolysis it was possible to prepare from it guanosine and adenosine.

CONCLUSIONS.

1. Methods are developed for separation of ribopolynucleotides from thymonucleic acid (desoxypentose nucleic acid).
2. Conditions are given for the isolation of the purine bases from ribonucleic acids, also conditions for the separation of the two bases.
3. The pancreas nucleic acid purified by the new process differed from the yeast nucleic acid in the content of ribose, of purine bases, and in the proportion of the two bases.
4. On partial hydrolysis adenosine and guanosine were prepared from this acid.

EXPERIMENTAL.

Preparation and Purification of the Barium Salts of the Pancreas Nucleic Acid.

The pancreas nucleoprotein and the protein-free barium salts of the nucleic acids were prepared according to the descriptions given in a previous work.[4] The protein-free barium salts contained a mixture of thymonucleic and pentose nucleic acids. These were separated by the glacial acetic acid method. The pentose nucleic acid settles out more readily from a protein-free solu-

[6] Buell, M. V., and Perkins, M. E., *J. Biol. Chem.*, **72**, 745 (1927).

tion than the thymonucleic acid; the latter can then be recovered together with some pentose nucleic acid from the mother liquors by precipitation with alcohol.

The purification of the pentose nucleic acid was followed by the fuchsin sulfurous acid test. This color test, introduced in nucleic acid chemistry by Feulgen,[7] can be used for quantitative purposes as well.[8] The amount of thymonucleic acid in a sample can be determined with fairly good accuracy as is shown by the following.

10 gm. of barium salt were suspended in 100 cc. of ice water and hydrochloric acid was added with stirring in a mortar until the nucleic acid formed a cake. This cake was repeatedly washed with cooled water, then suspended in about 50 cc. of water. A concentrated solution of sodium hydroxide was added in small portions under cooling with ice and stirring. Immediately after dissolution 1 liter of glacial acetic acid was added and the precipitate collected and dried on a Buchner funnel. The weight of the air-dry substance was 7.5 gm. By adding 1½ liters of alcohol to the mother liquor a second precipitate was obtained which also was dried and analyzed for comparison. The original material showed the presence of 3.3 per cent of thymonucleic acid. The glacial acetic acid precipitate contained less than 1 per cent and the substance obtained from the glacial acetic acid solution contained about 10 per cent.

The barium salts were therefore all purified by the above procedure. The resulting acid salts of the pentose nucleic acid thus obtained were insoluble in water but dissolved on the addition of alkali. They contained a negligible amount of thymonucleic acid, never more than 1 or 2 per cent. They showed, as a rule, a negative biuret test.

Analysis of Purified Pancreas Nucleic Acid.

In order to test the methods of analysis, parallel experiments were made on purified yeast and pancreas nucleic acids. The *yeast nucleic acid* was purified through its barium salt in the same

[7] Feulgen, R., *Z. physiol. Chem.*, **92**, 154 (1914).
[8] Widström, G., *Biochem. Z.*, **199**, 298 (1928).

manner as described for the purification of the pancreas nucleic acid. It analyzed as follows.

The air-dry material contained 12.54 per cent moisture.

0.1750 gm. dry substance: 17.97 cc. 0.1 N HCl (Kjeldahl).
0.2625 " " " : 0.0789 gm. $Mg_2P_2O_7$.
Found. N 14.37, P 8.37.

Three different samples of *pancreas nucleic acid* were used. All were purified by precipitation with dilute hydrochloric acid followed by a second precipitation with glacial acetic acid. Sample II was once more suspended in a small amount of ice water, treated with 10 per cent hydrochloric acid, washed with cold water, then dissolved by adding alkali, and reprecipitated with glacial acetic acid. The samples analyzed as follows.

Sample I.—The air-dry substance contained 9.06 per cent moisture.
0.100 gm. dry substance: 11.00 cc. 0.1 N HCl (Kjeldahl).
4.305 mg. " " : 25.52 mg. ammonium phosphomolybdate (Pregl-Lieb).
Found. N 15.40, P 8.60.
Sample II.—The air-dry substance contained 9.35 per cent moisture.
0.0907 gm. dry substance: 9.90 cc. 0.1 N HCl (Kjeldahl).
0.1361 " " " : 0.0400 gm. $Mg_2P_2O_7$.
Found. N 15.30, P 8.20.
After the second purification it analyzed as follows:
The air-dry substance contained 7.26 per cent moisture.
0.093 gm. dry substance: 10.61 cc. 0.1 N HCl (Kjeldahl).
0.139 " " " : 0.0424 gm. $Mg_2P_2O_7$.
Found. N 16.02, P 8.51.
Sample III.—The air-dry substance contained 6.80 per cent moisture.
0.0932 gm. dry substance: 10.69 cc. 0.1 N HCl (Kjeldahl).
0.1398 " " " : 0.0434 gm. $Mg_2P_2O_7$.
Found. N 16.05, P 8.65.

Pentose Analysis.

The Hoffman[9] micro method of pentose estimation applied to purine nucleotides gave values of furfuraldehyde corresponding to 80 per cent of the theoretical. Three analyses on crystalline guanylic acid gave 78.0, 81.2, and 79.5 per cent of the theoretical amount. Three analyses on crystalline cytidine phosphoric acid gave 12.6, 7.7, and 10.0 per cent of the theoretical amount of

[9] Hoffman, W. S., *J. Biol. Chem.*, **73**, 15 (1927).

pentoses. Hoffman found only 4.5 per cent of the amount in cytidine phosphoric acid but 13.7 per cent of the amount in uridine phosphoric acid. Hence the average yield of furfuraldehyde from the pyrimidine nucleotides corresponds to about 10 per cent of the pentoses present.

The mean value of eight analyses on the dry sample of yeast nucleic acid was 18.7 ± 0.32 per cent (the highest being 20.5 and the lowest 18.0). On the basis of the nucleic acid content of the sample the value corresponds to 21.6 per cent, whereas the theory requires 20.8 per cent.

The mean value of five analyses of Sample I of the pancreas nucleic acid was 21.5 ± 0.18 per cent of the dry substance and of four analyses of Sample II 21.0 ± 0.44 per cent. Calculated on ash-free nucleic acid (of the type of yeast nucleic acid), the figures are 23.9 and 24.4 per cent.

The theory requires for a tetranucleotide of the yeast nucleic acid type 20.8 per cent, for a pentose pentanucleotide 23.6 per cent, and for a hexanucleotide 25.6 per cent.

In this and in the following calculations the types of pentose polynucleotides considered are: (1) yeast nucleic acid, (2) a pentanucleotide containing yeast nucleic acid and an additional molecule of guanylic acid, (3) a hexanucleotide containing yeast nucleic acid and 2 additional molecules of guanylic acid. The phosphorus content in these three polynucleotides differs very slightly, being 9.54 per cent in yeast nucleic acid, 9.41 per cent in the pentanucleotide, and 9.34 per cent in the hexanucleotide.

Analysis of Purine Bases, Purine- and Pyrimidine-Nitrogen, and Easily Hydrolyzable Phosphates.

A. Hydrolysis with Sulfuric Acid.

1. Yeast Nucleic Acid. Hydrolysis I.—3 gm. of air-dry yeast nucleic acid were hydrolyzed with 40 cc. of a 5 per cent (by volume) sulfuric acid solution for 2½ hours in a water bath at 100°. After hydrolysis the precipitate of barium sulfate was centrifuged. The solution was made up to 50 cc. with water.

Determination of Purine Nitrogen.—Two aliquot samples of 7.5 cc. each were used. To each sample a hot solution of silver sulfate was added, until the filtrate gave no further precipitation

with this reagent. After cooling, the precipitate was centrifuged and washed twice with a silver sulfate solution. The purine bases were then dissolved in hot hydrochloric acid, the silver chloride washed with hot water, the liquids combined, and water added to bring the volume of the solution to 50 cc. 25 cc. of this solution contained 18.62 mg. of N (average of two determinations: 18.59 and 18.64). Hence, 37.24 mg. of N in 50 cc.

Since 7.5 cc. of the original solution contained 0.394 gm. of dry yeast nucleic acid with 56.7 mg. of nitrogen the amount of nitrogen in the purine fraction was 65.7 per cent of the total amount of nitrogen in the nucleic acid. Calculated for yeast nucleic acid 66.7 per cent of nitrogen or two-thirds of the total amount.

Separation of Purine Base.—34 cc. of the original solution were neutralized with a concentrated solution of sodium hydroxide and the purine bases separated according to the method outlined in the introduction.

Weight of dry guanine precipitate 0.227 gm. (N found, 39.99 per cent). Hence, 0.196 gm. of guanine.

Weight of air-dry adenine picrate 0.406 gm., containing 4.85 per cent water (calculated for 1 H_2O, 4.71 per cent). Hence, 0.386 gm. of dry picrate or 0.144 gm. of adenine.

34 cc. of the hydrolysate corresponded to 1.785 gm. of dry yeast nucleic acid, containing 8.37 per cent phosphorus. On the basis of the nucleic acid content of the sample the guanine content found corresponds to 12.52 per cent and the adenine content to 9.20 per cent of the free acid.

In this procedure the addition of ammonia as seen was omitted. Indeed, in blank experiments, where mixtures of guanine and adenine were used, it was found that guanine in this way settles out quantitatively. After neutralizing a guanine solution containing 1.000 gm. of guanine in 25 cc., 0.968 gm. were recovered. On the other hand, the adenine remained in the filtrate if the guanine precipitate was filtered off while the solution was still slightly warm, whereas if filtration was done some time after, a certain amount of adenine also precipitated.

Hydrolysis II.—Another sample of 3 gm. was hydrolyzed in the same way. The hydrolysate was diluted to 100 cc. 4 cc. were used for determining the phosphoric acid cleaved during hydrolysis and 96 cc. were used for determining the content of purine bases and the nitrogen in the pyrimidine bases.

Estimation of Free Phosphoric Acid Liberated during the Hydrolysis.—4 cc. of the hydrolysate were diluted to 20 cc. and the free phosphates were determined in 5 cc. samples after precipitation with magnesia mixture. The ammonium phosphomolybdate weighed 84.4, 82.0, and 85.6 mg. Mean 84.0 mg. = 1.20 mg. of P.

5 cc. of this solution corresponded to 0.0262 gm. of nucleic acid = 2.20 mg. of P. Hence, in the course of the hydrolysis 54.5 per cent of the phosphates contained in the yeast nucleic acid were cleaved. The calculated value is given in Table I, where Jones' factor[10] of correction for hydrolysis of the pyrimidine nucleotides is used.

Nitrogen Partition between the Purine and Pyrimidine Fractions.—96 cc. of the hydrolysate were treated with a silver sulfate solution as indicated above. The purine-free mother liquor contained 112.2 mg. of nitrogen. Since 96 cc. of the hydrolysate correspond to 0.360 gm. of total nitrogen, the amount in the pyrimidine bases was found to be 31.2 per cent, against the calculated amount 33.3 per cent or one-third of the total nitrogen content of the yeast nucleic acid.

The precipitate of the purine bases was dissolved in hot hydrochloric acid and the silver chloride washed and filtered off after cooling. The volume of the solution was 150 cc. To the hot solution ammonia was added in an excess of 2 per cent. The precipitate was collected after 24 hours and dried. The mother liquor was concentrated to 50 cc. No further precipitate of guanine was obtained on adding ammonia and letting stand for 12 hours.

Weight of dry guanine precipitate 0.2958 gm. (N found, 43.29 per cent). Hence, 0.276 gm. of guanine.

From the acidified mother liquor a picrate was obtained, which when dry weighed 0.817 gm. Hence, 0.303 gm. of adenine.

Since 96 cc. of the hydrolysate correspond to 2.522 gm. of dry yeast nucleic acid, the guanine content calculated on the free acid is 12.49 per cent and the adenine content 13.71 per cent. With the technique used in this experiment the value obtained for the adenine content is too high.

[10] Jones, W., Nucleic acids, London and New York, 2nd edition, 40 (1920).

2. Pancreas Nucleic Acid. Hydrolysis I.—3 gm. of air-dry pancreas nucleic acid (Sample II) were treated in the same way as described in Hydrolysis I with yeast nucleic acid. The volume of the hydrolysate was brought to 50 cc.

Nitrogen in Purine Fraction.—Two samples of 7.5 cc. each were treated with a silver sulfate solution in order to precipitate the purine bases. The amount of nitrogen in the precipitate with the purine bases was determined in exactly the same way as in the case of the yeast nucleic acid. 25 cc. of the final solution contained (first sample) 23.51 mg. of N and (second sample) 23.15 mg. of N. Mean 23.33 mg. of N. Hence 46.66 mg. of N in 50 cc.

Since 7.5 cc. of the hydrolysate corresponded to 0.4075 gm. of dry nucleic acid with 62.4 mg. of N, the amount of purine nitrogen found was 74.7 per cent of the total nitrogen content.

The calculated percentages of nitrogen in the purine bases in the different polynucleotides are given in Table I.

Phosphoric Acid Hydrolyzed.—1 cc. of the hydrolysate was diluted to 20 cc. The free phosphates were determined in two 5 cc. samples. Found 52.7 and 49.5 mg. of ammonium phosphomolybdate. Mean 51.1 mg. = 0.733 mg. of P.

5 cc. of this final solution corresponded to 0.0136 gm. of nucleic acid with 1.12 mg. of P; hence, 65.5 per cent of the phosphates in the nucleic acid were cleaved in the course of the hydrolysis.

Purine Bases.—The content of guanine and adenine was determined in 34 cc. of the hydrolysate in exactly the same way as in Hydrolysis I with yeast nucleic acid.

Weight of dry guanine 0.386 gm. (N found, 37.65 per cent). Hence, 0.314 gm. of guanine.

Weight of dry adenine picrate 0.259 gm. Hence, 0.096 gm. of adenine.

34 cc. of the hydrolysate corresponded to 1.85 gm. of dry nucleic acid with 8.20 per cent of phosphorus. On the basis of the nucleic acid content of the sample the guanine content in the free acid was 19.7 per cent and the adenine content 6.0 per cent.

Hydrolysis II.—3.960 gm. of air-dry substance (Sample III) were hydrolyzed with 60 cc. of a 5 per cent sulfuric acid solution. After removal of the barium sulfate the volume of the solution was diluted to 100 cc.

Phosphoric Acid Hydrolyzed.—3 cc. of the diluted hydrolysate

were made up to 25 cc. From two samples of 5 cc. each 83.0 and 84.8 mg. of ammonium phosphomolybdate were obtained on analysis according to the procedure described above. Mean 83.9 mg. of ammonium phosphomolybdate = 1.20 mg. of P.

In 1 cc. of the original solution the amount of phosphorus was 3.19 mg. and in 5 cc. of the final solution 1.91 mg. The amount hydrolyzed during $2\frac{1}{2}$ hours with 5 per cent sulfuric acid was 63 per cent, which is the calculated amount for a pentanucleotide with three purine nucleotides.

Pyrimidine Nitrogen and Purine Bases.—97 cc. of the hydrolysate were treated with silver sulfate as in Hydrolysis II with yeast nucleic acid. After removal of the purine bases 0.149 gm. of nitrogen were found in the combined mother liquors and washing fluids.

Since 97 cc. of the hydrolysate contained 0.576 gm. of nitrogen, the non-purine nitrogen in the sample was 25.9 per cent of the total amount present.

The calculated amount of nitrogen in the pyrimidine bases in the different polynucleotides is given in Table I.

The precipitate containing the purine bases was treated in exactly the same way as under Hydrolysis II with yeast nucleic acid. The purine bases were separated in a 2 per cent ammoniacal solution.

Weight of dry guanine 0.627 gm. (N found, 44.94 per cent). Hence, 0.607 gm. of guanine.

Weight of dry adenine picrate 1.019 gm. Hence, 0.378 gm. of adenine.

On the basis of the content of ash-free nucleic acid in the sample, the guanine content was 18.7 per cent and the adenine content 11.6 per cent. The latter figure is liable to the same error as the figure found in Hydrolysis II with yeast nucleic acid.

B. Hydrolysis with Methyl Alcoholic HCl.

1. Yeast Nucleic Acid. Hydrolysis I.—5 gm. of air-dry yeast nucleic acid were suspended in 150 cc. of dry methyl alcohol, and the suspension saturated with dry hydrogen chloride gas. The further procedure for the isolation and separation of the purine bases was the same as given in the introduction.

Weight of dry guanine 0.518 gm. Weight of dry adenine picrate 1.039 gm. Hence, 0.386 gm. of adenine.

The guanine content found was 13.5 per cent and the adenine content 10.1 per cent.

After removal of the purine hydrochlorides there was 0.1705 gm. of nitrogen in the methyl alcoholic solution. Since the whole sample contained 0.627 gm. of nitrogen, the amount in the methyl alcoholic mother liquor was 27.2 per cent of the total amount present.

Hydrolysis II.—Another sample of 5 gm. of air-dry yeast nucleic acid was treated in the same way as in Hydrolysis I.

Weight of dry guanine 0.470 gm. Weight of dry adenine picrate 1.058 gm. Hence, 0.393 gm. of adenine.

On the basis of the content of ash-free nucleic acid in the sample the percentage of guanine was 12.3 per cent and of adenine 10.2 per cent.

After removal of the purine hydrochlorides there was 0.1908 gm. of nitrogen in the methyl alcoholic mother liquor or 32 per cent of the total amount of nitrogen present.

2. Pancreas Nucleic Acid. Hydrolysis I.—5 gm. of air-dry substance (Sample III) were hydrolyzed in the same way as described for the yeast nucleic acid.

Weight of air-dry guanine 0.910 gm. (N found, 42.36 per cent). Hence, 0.831 gm. of guanine.

Weight of dry picrate 0.628 gm. (N found, 29.83 per cent). Hence, 0.611 gm. of adenine picrate or 0.228 gm. of adenine.

On the basis of the content of ash-free nucleic acid in the sample (calculated as a tetranucleotide), the guanine content was 19.7 and the adenine content 5.4 per cent.

After removal of the purine hydrochlorides there was 0.1629 gm. of nitrogen in the methyl alcoholic mother liquor. Since the sample contained 0.733 gm. of nitrogen, the nitrogen in the mother liquor was 22.2 per cent of the total amount present.

Hydrolysis II.—4 gm. of air-dry substance (Sample II, purified) were hydrolyzed and the analysis performed as in Hydrolysis I.

Weight of dry guanine 0.764 gm. Weight of dry adenine picrate 0.400 gm. Hence, 0.149 gm. of adenine.

The guanine content calculated on the free acid was 27.1 per cent and the adenine content 4.50 per cent.

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After removal of the purine chlorides there was 0.143 gm. of nitrogen in the methyl alcoholic mother liquor. Since the total amount of nitrogen in the sample was 0.595 gm., the nitrogen in the mother liquor was 24 per cent of the total amount present.

Nucleosides.

14.0 gm. of the pancreas ribopolynucleotides were dissolved in 75 cc. of a 5 per cent ammonia solution and heated in autoclaves for 4 hours, the inner temperature being maintained at 135°. On cooling, the reaction product completely gelatinized. The gelatinous precipitate was used for the preparation of guanosine, and the filtrate for that of adenosine.

The *guanosine* fraction was redissolved and the guanosine purified through the lead salt in the usual manner. The yield of the crystalline material was 1.5 gm. A parallel experiment with yeast nucleic acid yielded 1.0 gm. of guanosine.

0.0443 gm. substance: 7.85 cc. 0.1 N HCl.

$C_{10}H_{13}N_5O_5$.	Calculated.	N 24.73.
	Found.	" 24.76.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-1.22° \times 100}{1 \times 2} = -61.0° \text{ (in water).}$$

The filtrate from guanosine was acidulated with sulfuric acid to slightly acid reaction to Congo red and an excess of picric acid solution was added. A small precipitate formed which was removed by filtration and on cooling of the filtrate, a light yellow gelatinous precipitate formed. The weight of the air-dry picrate was 2.137 gm. It was recrystallized from water and analyzed as follows:

0.0486 gm. substance: 7.87 cc. 0.1 N HCl (Kjeldahl).[11]

$C_{10}H_{13}N_5O_4 \cdot C_6H_2(NO_2)_3OH$.	Calculated.	N 22.58.
	Found.	" 22.67.

[11] Elek, A., and Sobotka, H., *J. Am. Chem. Soc.*, **48**, 501 (1926).

ON THE STRUCTURE OF MELIBIOSE.

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Very strong evidence has been advanced in favor of the theory postulating the structure of melibiose as 6-α-galactosido-glucose <1,5>. There has been a general feeling in recent years among sugar chemists that the evidence furnished by the methylation method such as used by Haworth[1] and coworkers in connection with melibiose needs substantiation by the simpler methods. True, in this case Helferich and Bredereck[2] succeeded by a very ingenious method in the synthesis of the disaccharide, but the yield of the product was so small that the authors did not obtain the substance in a state of absolute purity. New and good evidence in favor of the above structure was recently furnished by Josephson.[3]

It remained to reconcile the observations of Levene and Wintersteiner[4] with the view of the workers mentioned above. The method introduced by Levene was tested successfully on several occasions by Levene and coworkers,[5] also more recently by Heidelberger and Kendall[6] and there was no reason to assume that it would fail in the case of melibiose.

* Fellow of The Rockefeller Foundation.

[1] Haworth, N. H., Loach, J. V., and Long, C. W., *J. Chem. Soc.*, 3146 (1927).

[2] Helferich, B., and Bredereck, H., *Ann. Chem.*, **465**, 166 (1928).

[3] Josephson, K., *Ann. Chem.*, **472**, 230 (1929).

[4] Levene, P. A., and Wintersteiner, O., *J. Biol. Chem.*, **75**, 315 (1927).

[5] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **65**, 31 (1925). Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, **71**, 471 (1926–27). Levene, P. A., and Wolfrom, M. L., *J. Biol. Chem.*, **77**, 671 (1928). Levene, P. A., and Mori, T., *J. Biol. Chem.*, **81**, 215 (1929).

[6] Heidelberger, M., and Kendall, F. E., *J. Biol. Chem.*, **84**, 639 (1929).

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We therefore decided to test the structure of melibiose by methods which had not as yet been applied to the study of this disaccharide. On the basis of the 6-galactosido structure, the glucose radical of the melibiose should have the hydroxyls (3), (4), and (5) free. The condition of hydroxyls (4) and (5) can be easily tested by the formation or non-formation of either <1,4> or <1,5> glucosides and furthermore, by the conduct of the glucal radical.[7] When hydroxyl (4) is free as in the case of glucose, rhamnose,[8] and gentiobiose,[7] then the glucals give a typical pine splinter color test. If this hydroxyl is substituted as in lactose[9] and cellobiose,[10] then the test is negative.

In the case of melibiose, it was possible to prepare the two glucosides. The <1,4> glucoside was prepared by allowing the disaccharide to stand 24 hours at room temperature in a solution of dry methyl alcohol containing 0.5 per cent of hydrogen chloride gas. In an aqueous solution of 0.1 N hydrochloric acid, this glucoside hydrolyzed at the temperature of a boiling water bath to the extent of 90 per cent within 10 minutes.

The <1,5> glucoside was prepared through the bromoheptaacetyl melibiose. The acetyl methylmelibioside was obtained in crystalline form; the free glucoside in a chemically pure, though amorphous state. This glucoside showed the usual resistance to hydrolysis characteristic of the <1,5> glucosides.

The melibial was prepared from the same 1-bromoheptaacetyl melibiose. The acetyl melibial was obtained crystalline and the free melibial in an amorphous form. The free as well as the acetyl melibial gave the characteristic pine splinter test, thus further showing that the hydroxyl in position (4) is not substituted in melibiose.

With the evidence obtained in this manner it was necessary to repeat the experiment of Levene and Wintersteiner[4] on melibionic acid. These authors had observed the formation of a <1,5> lactone and failed to observe the formation of the <1,4> lactone. The experiment was repeated under conditions similar to those of Levene and Wintersteiner, save that the

[7] Bergmann, M., and Freudenberg, W., *Ber. chem. Ges.*, **62**, 2783 (1929).
[8] Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 440 (1921).
[9] Fischer, E., and Curme, G. O., *Ber. chem. Ges.*, **47**, 2047 (1914).
[10] Fischer, E., and von Fodor, K., *Ber. chem. Ges.*, **47**, 2057 (1914).

polariscope used was sensitive to 0.002 of a degree. The formation of the <1,5> lactone formerly observed was substantiated. It was, however, observed that if the experiment were allowed to continue longer than in the case of Levene and Wintersteiner, the formation of the <1,4> lactone began to be noticeable. After 6 days, the <1,4> lactone formation was demonstrable not only polariscopically but also by titration.

All these findings, then, are in harmony with the theory of the 6-α-galactosido-glucose <1,5> structure of melibiose and are further evidence that the rate of lactone formation is a useful and convenient method in the study of the structure of polysaccharides.

EXPERIMENTAL.

Preparation of Melibiose.

The melibiose was prepared from air-dry raffinose ($[\alpha]_D^{20}$ = +103.5°, in water) through fermentation with bakers' yeast as described by Levene and Wintersteiner.[4]

Twice recrystallized from 50 per cent alcohol the melibiose was ash-free. It reduced Fehling's solution.

8.180 mg. air-dry substance lost on drying under reduced pressure (80°) 0.760 mg.

7.060 mg. air-dry substance lost on drying under reduced pressure (80°) 0.650 mg.

$C_{12}H_{22}O_{11} \cdot 2\ H_2O$. Calculated. H_2O 9.53.
Found. " 9.28, 9.20.

The air-dry substance, dissolved in water, showed a slight upward mutarotation with the following equilibrium value.

$$[\alpha]_D^{20} = \frac{+2.60° \times 100}{1 \times 2} = +130°.$$

Oxidation to Melibionic Acid.

Melibiose was oxidized with bromine in the presence of barium benzoate as recommended by Hudson and Isbell.[11]

10 gm. of air-dry melibiose (0.026 mol) were dissolved in 300 cc. of water containing 12.5 gm. of crystalline barium hydroxide and

[11] Hudson, C. S., and Isbell, H. S., *J. Am. Chem. Soc.*, **51**, 2225 (1929).

9.7 gm. of benzoic acid (0.080 mol) and allowed to stand at room temperature. After 36 hours the reducing power to Fehling's solution disappeared. The excess of bromine was then removed by aeration. The hydrogen bromide was removed by silver carbonate and the excess of silver by hydrogen sulfide and the benzoic acid by shaking with chloroform. The sulfuric acid was removed quantitatively by means of barium hydroxide and the resulting clear filtrate was boiled with calcium carbonate. The filtrate was concentrated to a small volume and treated with an excess of methyl alcohol. The precipitate was dissolved in a

TABLE I.

Changes in Rotation of Melibionic Acid Solution.

l = 2 dm. t = 25° λ = 5829 Å.

Time.	α_D^{25}	Time.	α_D^{25}
hrs.	*degrees*	*hrs.*	*degrees*
½	6.822	10	7.024
¾	6.870	12	7.035
1	6.913	24	7.116
1½	6.946	29	7.160
2	6.958	38	7.205
2½	6.962	48	7.232
3½	6.978	72	7.293
4½	6.983	86	7.310
8	7.016	126	7.331

little water to which 1½ volumes of methyl alcohol were added. On cooling the calcium salt crystallized (6.6 gm.). It had no reducing power but was not analytically pure. Hence, it was redissolved, a slight excess of calcium hydroxide was added, and the excess removed by means of carbon dioxide gas. The clear solution was concentrated and treated with an excess of methyl alcohol. The precipitate was again dissolved in a little water, centrifuged to remove traces of calcium carbonate, and to the clear supernatant solution methyl alcohol was added.

5.255 mg. dry substance: 7.330 mg. CO_2 and 2.660 mg. H_2O.
9.860 " " " : 1.77 mg. $CaSO_4$.
$(C_{12}H_{21}O_{12})_2Ca$. Calculated. C 38.18, H 5.61, Ca 5.31.
Found. " 38.03, " 5.66, " 5.28.

Mutarotation and Titration Experiments with Melibionic Acid.

1.0000 gm. of air-dry calcium melibionate containing 15.23 per cent of water, thus corresponding to 0.8477 gm. of dry substance or to 0.8050 gm. of free melibionic acid was dissolved in 23.00 cc. of 0.1 N hydrochloric acid (calculated amount 22.38 cc.) and the volume made up to 25 cc. with water. The change in rotation of the solution was followed during a period of 6 days, the temperature being kept constant all the time (25°). The changes in rotation are given in Table I and in Figs. 1 and 2.

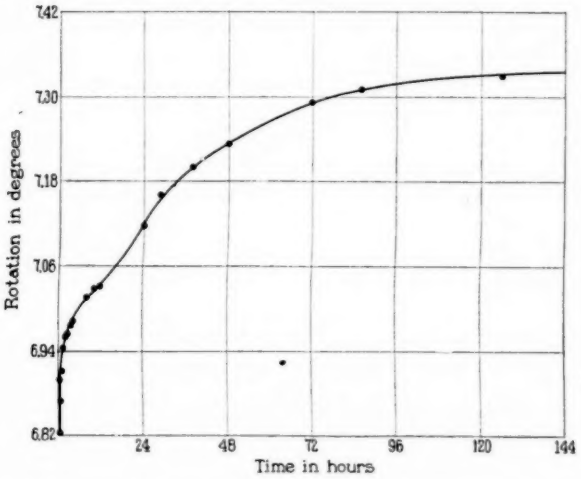

FIG. 1. Changes in rotation of melibionic acid. This curve shows the very slow course of the <1,4> lactone formation.

At the end of the experiment the solution showed no reduction towards Fehling's solution; hence, no hydrolysis had taken place.

Two samples of 5 cc. each were titrated with a 0.1 N sodium hydroxide solution with phenolphthalein as indicator. They were neutralized by 4.00 and 4.05 cc. of 0.1 N NaOH. At this stage 2 cc. of 0.1 N alkali were added and the solution was heated and titrated back with 0.1 N HCl. 1.30 and 1.25 cc. of the acid were required for neutralization. Hence, the total amount of melibionic acid was equivalent to 4.70 and 4.80 cc. of 0.1 N alkali

(4.6 cc. is the value required by theory), of which 0.70 and 0.75 cc., or 14.9 and 15.8 per cent, are equivalent to the acid in lactone form.

The formation of the stable <1,4> lactone, indicated both by the changes in the rotatory power of the solution of the melibionic acid and by the titration experiment, was followed in two other experiments. In one, hydrochloric acid was added to the solution of the calcium salt in a quantity equivalent to 89 per cent of the salt and in the other, an excess of the acid was added to make the total concentration of the hydrochloric acid 0.1 N.

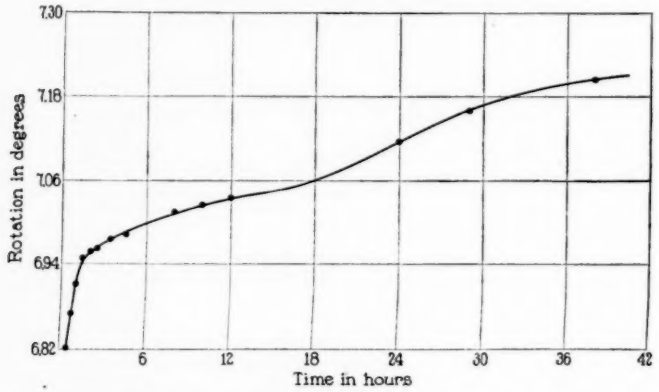

FIG. 2. Changes in rotation of melibionic acid. This curve shows the rapid decrease in the formation of the <1,5> lactone at the end of the 2nd hour and the beginning of the <1,4> lactone formation after about 20 hours.

In both cases it was possible to follow the lactone formation by titration.

Two samples of 0.2000 gm. of the air-dry calcium salt were dissolved in a final volume of 5 cc. To Sample A 4 cc. of 0.1 N HCl were added (calculated 4.47 cc.) and to Sample B 4.5 cc. of 0.1 N and 0.5 cc. of 1.0 N HCl. 1 cc. samples were titrated with 0.1 N NaOH solution.

Immediately.....	Sample A. 0.78 cc.	Sample B. 1.83 cc.
After 48 hrs.....	" " 0.715 "	" " 1.75 "

The lactone formed was somewhat less than 10 per cent of the melibionic acid.

Preparation of Melibiose Octaacetate.

Samples of 25 gm. of air-dry melibiose were suspended in 125 cc. of dry pyridine and 90 cc. of acetic anhydride were added without cooling. The substance dissolved rather quickly with a considerable rise of temperature. It was allowed to stand at room temperature for 18 hours. The solution was then poured into 1.5 liters of ice water with stirring. The acetate settled out as a white flocculent mass. It was collected on a Buchner funnel, again suspended in ice water, and filtered. It was recrystallized from hot 98.5 per cent alcohol. The recrystallized substance became less soluble in alcohol. The yield of three times recrystallized substance was from 28 to 35 gm. in different samples (62 to 74 per cent of the theory).

Recrystallized from alcohol the acetate did not contain any water of crystallization and had a melting point of 177.5–178° (not corrected), 177.5° (corrected) Hudson.[12] After three recrystallizations a sample had the following specific rotation.

$$[\alpha]_D^{23} = \frac{+4.18° \times 100}{1 \times 4} = +104.5° \text{ (in chloroform).}$$

After five recrystallizations the rotation was

$$[\alpha]_D^{27} = \frac{+4.80° \times 100}{1 \times 4.64} = +103.5°.$$

The value given by Hudson for the β form of melibiose octaacetate is +102.5°.

0.2000 gm. substance on shaking for 8 hours with an excess of 0.1 N NaOH neutralized 24.15 cc. of the solution.

0.2000 gm. of another sample neutralized 23.79 cc. of the solution.

Calculated. $C_{28}H_{38}O_{19}$. 23.59 cc.

Acetobromomelibiose.

The octaacetate (35 gm.) was dissolved in hot acetic anhydride (50 cc.), the solution cooled to 10°, and mixed with a saturated solution of hydrobromic acid in glacial acetic acid (100 cc.). The solution was allowed to stand for 2 hours at room tempera-

[12] Hudson, C. S., and Johnson, J. M., *J. Am. Chem. Soc.*, **37**, 2752 (1915).

ture. The solution remained colorless and no rise in temperature was observed. It was poured into ice water under vigorous stirring and the precipitate taken up in chloroform. The chloroform solution was washed several times with ice water. It was dried over calcium chloride and sodium sulfate. The chloroform was removed by evaporation and the residue was allowed to stand in a vacuum desiccator over phosphorus pentoxide. It was obtained as a white powder. After dissolving in absolute alcohol or ether it could easily be frozen out at the temperature obtained by ether and solid carbon dioxide but the substance did not crystallize in spite of repeated precipitations in the cold. The amorphous material was used for the preparation of β-methyl melibioside <1,5> heptaacetate and for the reduction to melibial hexaacetate.

The acetobromomelibiose was quite stable and showed no signs of decomposition on standing for several days.

The bromine content was determined in two samples. 0.530 gm. of substance dried *in vacuo* over P_2O_5 was dissolved in a small volume of methyl alcohol and a concentrated solution of sodium hydroxide added. On titration the sample consumed 14.1 cc. of 0.05 N $AgNO_3$ solution. 0.300 gm. of another sample required 8.6 cc. of 0.05 N $AgNO_3$ solution.

$C_{26}H_{33}O_{17}Br$. Calculated. Br 11.43.
Found. " 10.6, 11.4.

Melibial Hexaacetate.

21 gm. of acetobromomelibiose were added in small portions to 210 cc. of a 50 per cent acetic acid solution containing 42 gm. of zinc dust. The reaction was allowed to proceed for 3 hours with constant stirring and cooling. The zinc dust was then filtered off and suspended in a mixture of water and ice. The acetic acid solution was neutralized with solid bicarbonate under cooling and then extracted with chloroform. The chloroform solution was washed with ice water and dried in the usual way. During the evaporation of the chloroform the melibial hexaacetate crystallized in the distilling flask. In one experiment the acetate separated out in the course of the reaction and the major part of the reaction product was filtered off with the zinc dust.

The acetate was recrystallized four times from absolute alcohol and showed a constant rotation and a definite melting point. It melted at 113° (not corrected). A qualitative test for bromine was negative.

On drying *in vacuo* at 100°, it did not lose in weight.

$$[\alpha]_D^{21} = \frac{+3.48° \times 100}{1 \times 4} = +87.0° \text{ (in chloroform).}$$

The dry substance analyzed as follows:

5.385 mg. substance: 10.210 mg. CO_2 and 2.800 mg. H_2O.
$C_{26}H_{32}O_{15}$. Calculated. C 51.4, H 5.76.
Found. " 51.7, " 5.82.
0.300 gm. neutralized in 4 hours 32.6 cc. of 0.1 N NaOH solution.
Calculated. 32.1 cc.
0.200 " " " 24 hours 21.5 cc. of 0.1 N $Ba(OH)_2$ solution.
Calculated. 21.4 cc.

Two samples of 0.200 gm. each were dissolved in a small amount of chloroform and 5 cc. of a 0.1848 N bromine solution in chloroform were added to each. The excess of bromine was titrated with a 0.01 N thiosulfate solution in the presence of potassium iodide, one sample after 5 minutes and the other after 20 minutes. On back titration 20.9 cc. of the thiosulfate solution were used for one sample and 20.7 cc. for the other.

Addition of bromine, expressed in 0.1 N solution: 7.15 and 7.17 cc.
Calculated. 7.13 cc.

The melibial hexaacetate showed a distinctly positive pine splinter reaction. For comparison the same reaction was made simultaneously with crystalline lactal specially prepared for this purpose. The latter compound gives a negative reaction.

Deacetylation of Melibial Hexaacetate.

For deacetylation, ammonia in methyl alcoholic solution was used as recommended by Bergmann.[13] The methyl alcohol was removed by distillation and the residue dried under diminished pressure at room temperature. It was dissolved in hot 98.5

[13] Bergmann, M., *Ann. Chem.*, **434**, 79 (1923).

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per cent ethyl alcohol and the melibial precipitated by adding several volumes of hot ethyl acetate, in which the acetamide is soluble. The dry substance was repeatedly extracted with ethyl acetate. The substance could not be crystallized. Dried under reduced pressure at 55°, it analyzed as follows:

5.110 mg.: 8.745 mg. CO_2 and 3.140 mg. H_2O.
$C_{12}H_{20}O_9$. Calculated. C 46.73, H 6.54.
Found. " 46.66, " 6.87.

To 0.1449 gm., dissolved in water, 3 cc. of a 0.3785 N solution of bromine in chloroform were added and the mixture was shaken for 2 minutes. On titration with a 0.0203 N thiosulfate solution after addition of potassium iodide, 9.90 cc. of the thiosulfate solution were consumed.

Addition of bromine, expressed in 0.1 N bromine solution: 9.35 cc.
Calculated. 9.40 cc.

$$[\alpha]_D^{25} = \frac{+5.69° \times 100}{2 \times 2} = +142.3° \text{ (in water).}$$

The substance gave a distinctly positive pine splinter test.

β-Methyl Melibioside <1, 5> Heptaacetate.

11.5 gm. of the acetobromomelibiose were dissolved in 120 cc. of methyl alcohol distilled over sodium and 12 gm. of dry silver carbonate added. At room temperature no reaction took place. Even on refluxing on a water bath the liberation of bromine proceeded very slowly. In one preparation the solution was found to be free of bromine after 12 hours, whereas the boiling in another preparation had to be continued for 60 hours. The silver precipitate was then removed and washed with dry methyl alcohol. On evaporation of the solution the methyl melibioside heptaacetate crystallized. It was recrystallized from absolute alcohol. The yield of twice recrystallized acetate was 5 gm.

This sample melted at 147° (not corrected) and showed a trace of reducing power. It was therefore recrystallized five times more from absolute alcohol. The melting point and the rotatory power were constant in the three last recrystallizations. There was no more trace of reduction towards Fehling's solution. Melting point 150° (not corrected).

$$[\alpha]_D^{22} = \frac{+1.81^\circ \times 100}{1 \times 2} = +90.5^\circ \text{ (in chloroform).}$$

Recrystallized from alcohol the sample did not contain any water of crystallization. It analyzed as follows:

4.885 mg. dry substance: 8.935 mg. CO_2 and 2.580 mg. H_2O.
0.1440 gm. " " : 0.0506 gm. AgI (Zeisel).
$C_{27}H_{38}O_{18}$. Calculated. C 49.83, H 5.89, OCH_3 4.74.
Found. " 49.87, " 5.91, " 4.64.

On shaking with a barium hydroxide solution in slight excess during 24 hours and back titration, it was found that

0.220 gm. neutralized 23.5 cc. of 0.1 N $Ba(OH)_2$ solution.
0.220 " " 23.4 " " 0.1 " " "
Calculated. 23.7 cc.

β-Methyl Melibioside <1,5>.

The acetate was suspended in a 0.2 N $Ba(OH)_2$ solution, an excess of 25 per cent being used, and shaken for 20 hours at room temperature. The excess of barium hydroxide was removed by carbon dioxide and the solution evaporated to dryness under reduced pressure. The dry residue was extracted with absolute ethyl alcohol. The glucoside was then precipitated by addition of ether to the alcoholic solution. Only an amorphous product was obtained. Dried at 55° under reduced pressure, it analyzed as follows:

4.780 mg. substance: 7.725 mg. CO_2 and 3.005 mg. H_2O.
0.1012 gm. " : 0.0630 gm. AgI (Zeisel).
$C_{13}H_{24}O_{11}$. Calculated. C 43.82, H 6.79, OCH_3 8.71.
Found. " 44.07, " 7.05, " 8.21.

$$[\alpha]_D^{27} = \frac{+1.50^\circ \times 100}{1 \times 2} = +75.0^\circ \text{ (in water).}$$

Preparation of the Methyl Melibioside <1,4>.

3 gm. of air-dry melibiose were suspended in 50 cc. of dry methyl alcohol, containing 0.5 per cent of dry hydrogen chloride. The sugar dissolved rapidly. The solution was allowed to stand for 24 hours at room temperature. During the last 12 hours there was no change in the rotatory power of the solution and the

reaction product did not reduce Fehling's solution. The solution was then shaken with silver carbonate. The precipitate was washed with dry methyl alcohol and filtered off. The mother liquor gave a negative test for chlorides. It was concentrated under diminished pressure to dryness. The residue was then taken up in hot absolute alcohol, from which the glucoside was precipitated by amyl alcohol. From the mother liquor a second

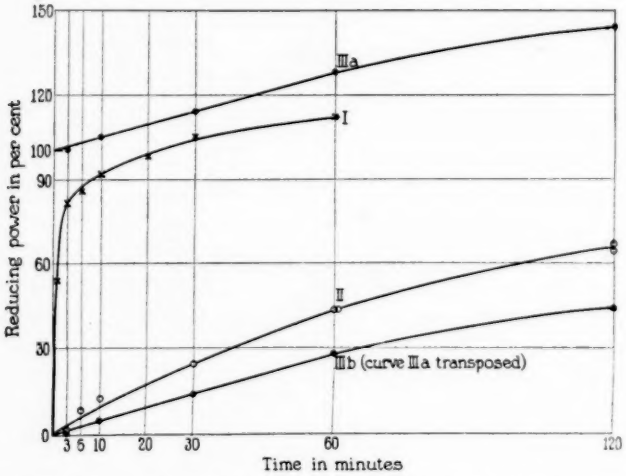

FIG. 3. Rates of hydrolysis of methyl melibioside <1,4> and of β-methyl melibioside <1,5>.

precipitate was formed after the addition of ether. It was not possible to obtain the glucoside in crystalline form. Both samples were dried under reduced pressure over phosphorus pentoxide at room temperature. They showed a trace of reduction to Fehling's solution.

The first sample analyzed as follows:

5.100 mg. substance (dried): 8.250 mg. CO_2 and 3.245 mg. H_2O.
0.0724 gm. " " : 0.0484 gm. AgI (Zeisel).

$C_{13}H_{24}O_{11}$.	Calculated.	C 43.82, H 6.79, OCH_3 8.71.
	Found.	" 44.11, " 7.11, " 8.82.

$$[\alpha]_D^{29} = \frac{+4.28^\circ \times 100}{2 \times 2} = +107.0^\circ \text{ (in water)}.$$

Hydrolysis of the Two Methyl Melibiosides.

The melibiosides were dissolved in 0.1 N HCl. 1 cc. of the solution contained an amount of glucoside dried *in vacuo* over P_2O_5 which corresponded to 1 mg. of melibiose. Samples of 2 cc. were hydrolyzed in sealed glass tubes in a boiling water bath. The time of hydrolysis is given in Fig. 3. Immediately after pipetting off the solutions they were frozen with ether and solid carbon dioxide and the same was done at the end of the hydrolysis. After hydrolysis 2 cc. of a 0.1 N NaOH solution were added to each tube. Sugar determinations were made by the method of Hagedorn and Jensen on samples of 0.5 cc. of this solution; thus containing an amount of glucoside corresponding to 0.25 mg. of dry melibiose.

The reducing power of the melibiose before and after hydrolysis was determined in exactly the same way.

The results are presented in Fig. 3.

If the reducing power of the melibiose is taken for 100 the increase due to hydrolysis of the disaccharide in the 0.1 N HCl solution can be seen in Fig. 3 (Curve IIIa). Curve I shows the rapid hydrolysis of the methyl melibioside <1,4> which hydrolyzes to 90 per cent within 10 minutes. On the other hand, the hydrolysis of the β-methyl melibioside <1,5> is very slow (Curve II). In order to show the influence of the hydrolysis of the disaccharide on the reducing power of the hydrolyzed methyl melibioside <1,5>, Curve IIIa is plotted as Curve IIIb from the same origin as Curve II.

CALCIUM AND PHOSPHORUS METABOLISM IN RATS DURING PREGNANCY AND LACTATION AND THE INFLUENCE OF THE REACTION OF THE DIET THEREON.

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(Received for publication, December 30, 1929.)

From a quantitative standpoint calcium and phosphorus occupy a foremost place in the list of mineral elements needed by the vertebrate animal organism. The necessity for an adequate supply of these elements is particularly striking during the period of growth and during the lactation period. In pregnancy, the normal adult female, which, prior to the onset of this condition, may be in calcium and phosphorus equilibrium, is required to build new tissue for the fetus and at parturition must secrete milk for the nourishment of the young. Up to the age of weaning, all the food elements obtained by the offspring are derived from the mother's milk.

Experimental work dealing with calcium and phosphorus metabolism during pregnancy and lactation has been carried out chiefly on cows. A few experiments on humans have also been reported. It is well known that heavy producing cows are generally in negative calcium and phosphorus balance. Thus Forbes and his coworkers (1) found that in forty-nine balance trials during liberal milk production without exception all of his animals were in negative calcium balance. Supplementing the diet with additional calcium did not alter the negative balances, indicating that the ability of the animal to absorb calcium is limited. The calcium balances changed from negative to positive during the period of pregnancy when the cows were dry. The phosphorus balances were in most cases negative but not invariably so, indicating that the conditions for the assimilation of this

element must be more readily attained than in the case of calcium. In general, the magnesium balances paralleled the calcium balances but not consistently so.

Partial studies on the mineral metabolism of pregnant women have also been reported. Landsberg (2) concludes that from the 2nd to the 9th month of pregnancy there was a positive balance of calcium, phosphorus, and magnesium in his subjects. The storage was in excess of that which he calculated for the content of the fetus. Hoffström's (3) experiments carried out over a longer period point in the same direction.

While the data obtained on cows and on women definitely indicate the need for calcium and phosphorus during pregnancy and lactation, they are, however, incomplete, in that the balance experiments were necessarily carried out during intermittent periods over the period of pregnancy and lactation. No analyses of the offspring at the time of weaning were made. The data of Forbes and his coworkers, while covering the entire period of lactation and pregnancy, were obtained over intermittent periods rather than during the entire cycle of one animal.

The difficulties inherent in experimental work of this type on large animals are apparent. We have sought to obtain a complete picture of the metabolism of calcium and phosphorus by carrying out experiments on animals in which it is possible to follow the calcium and phosphorus balances during the entire period of pregnancy and lactation and also to follow the balances during several such periods without intermission. We have found that the rat is well suited for experiments of this type. The period of pregnancy is comparatively short, the onset of pregnancy can be definitely ascertained, the lactation period is likewise of short duration, the calcium and phosphorus content of the young at the time of weaning can be determined, the experimental animals can be kept for long periods of time on a constant diet, and the excreta can be easily collected.

Since the experiments yielded the information that particularly during the lactation period the dietary requirements of the rat for calcium and phosphorus are increased, we have used the opportunity to study the effect of varying the reaction of the diet upon the absorption of these elements by both mother and offspring.

The relation of the reaction of the diet to the assimilation and

excretion of calcium and phosphorus has also received some experimental attention. Givens and Mendel (4) concluded that the addition of acid or base produces no significant effect upon the balance of calcium and phosphorus in dogs but the addition of hydrochloric acid increased the output of urinary calcium. Sato (5) found that the addition of sodium bicarbonate to milk had an unfavorable influence upon the deposition of calcium in the infant while Zucker (6) experimenting on an adult man found that acid causes a shift of both calcium and phosphorus from feces to urine while the reverse effect was obtained with sodium bicarbonate. The total balance was, however, not changed. Bogert and Kirkpatrick (7) believe that calcium is retained more readily on a base-forming diet than on an acid or neutral diet and that the calcium excretion is greater on an acid-forming diet than on a base-forming diet. The experiments were carried out on human subjects. Clark's (8) experiments on adult men showed that the storage of calcium in his subjects was not dependent upon the reaction of the diet supplied nor was it found possible to increase materially the retention of calcium by supplementing the diet with milk or calcium lactate. The inability of Hart, Steenbock, Teut, and Humphrey (9) to maintain calcium equilibrium in a liberally milking cow by supplemental hay is of interest in this connection.

Experimental Methods.

Adult female rats from the Division of Anatomy were used. Full grown animals were chosen since in these calcium and phosphorus storage, as a factor of growth, had ceased. The animals were kept in individual cages and were made thoroughly accustomed to their environment before any experimental work was begun. The composition of the diets is given in Table I. All of the essential ingredients known to be necessary for the nutrition of the rat were included. Diets I and II are normal natural foods to which sodium chloride and calcium carbonate were added. Diet III was made acid in reaction by replacing the calcium carbonate by calcium chloride and adding hydrochloric acid. Diet IV was rendered basic by addition of sodium bicarbonate. The butter fat was added fresh from time to time to a quantity of the stock diet and each new batch was analyzed for calcium and

phosphorus. Diet 228 is a purified diet[1] free from vitamin E prepared according to Evans and Burr (10).

The animals were kept in galvanized wire cages with removable screen floors. The food was placed in a cup of the side arm which also carried a trap to catch food which might be spilled. The excreta were collected in a porcelain dish placed beneath the bottom of the cage. After the birth of the young the porcelain

TABLE I.

Composition of Diets.

Diet No.	I	II	III	IV
Ground whole wheat	67.7	51.0	52.0	53.0
Wheat germ		10.0	10.0	10.0
Alfalfa leaf meal powder		5.0	5.0	5.0
Whole milk, powder	10.0	10.0	10.0	10.0
Casein	14.8	14.75	14.6	12.7
Butter fat	5.0	7.0	7.0	7.0
$CaCO_3$, U.S.P., ppt.	1.5	1.25		1.3
$CaCl_2$, C.P.			1.35	
HCl			0.05	
NaCl	1.0	1.0		
$NaHCO_3$, C.P.				1.0
Alkalinity, cc. 0.1 N NaOH per 100 gm.	300*	306†		440‡
Acidity, cc. 0.1 N HCl per 100 gm.			206	
Ca, mg. per 100 gm.	820	740	756	752
P, " " 100 "	550	500	530	530

* Alkalinity due to 1.5 per cent $CaCO_3$ added.

† Alkalinity due to 1.25 per cent $CaCO_3$ added = 250 cc. 0.1 N NaOH per 100 gm. of diet.

‡ Alkalinity due to 1.0 per cent $NaHCO_3$ = 119 cc. 0.1 N NaOH per 100 gm. of diet. Alkalinity due to 1.3 per cent $CaCO_3$ = 260 cc. 0.1 N NaOH per 100 gm. of diet.

dish was replaced by a glass crystallizing dish, the wire screen floor was removed and a screen of smaller mesh was placed on a supporting ring near the bottom of the glass dish. In this way the young were unable to gain entrance to the feed cups. All cages were kept in a constant temperature compartment ventilated by a forced draught of fresh air. The excreta were transferred to

[1] Diet 228 consisted of lard 7.7, casein 27, corn-starch 59.3, Salt Mixture 185 4.0, cod liver oil 2.0, and yeast 0.6 gm. daily.

evaporating dishes at the conclusion of each period and were dried on the steam bath after the addition of sodium carbonate to render the ash distinctly basic. No attempt was made to separate the feces for a given period by means of a marker since the periods were long and followed each other without intermission. No attempt was made to segregate the excreta of the young from those of the mother. The excreta of the young constitute a small part of the total collection and moreover in a balance between intake and output such a segregation is not necessary.

The estrous cycle of each rat was followed by daily examination of the vaginal smear according to the technique described by Long and Evans (11). Following the normal or rest period and when it was evident by the appearance of the vaginal smear that the animal would accept the male during the following 24 hours, she was placed in a cage with the male and examined on the following morning for the vaginal plug and the occurrence of spermatazoa. If this sign of copulation was found the female was returned to her cage. A small error is introduced by the intermission of 1 day but since the female is returned to her cage 24 hours after she was taken from it and since her diet was not changed it may be assumed that the excretion will nearly balance the intake during this time and that the error in a 22 day period will be small.

After the animal's return to the metabolism cage the vaginal smear was again examined daily and the characteristic erythrocyte sign of pregnancy (10) looked for on the 13th, 14th, and 15th day of gestation. The lactation period was continued for 18 to 21 days, at which time the young were usually sufficiently active to climb into the food cup. They were then removed, killed, and subjected to analysis. The mother was continued on the diet during a rest period and whenever possible again bred so that the balances could be followed over another cycle.

The food and excreta were ashed according to standard procedure. Calcium was determined by McCrudden's method and phosphorus by the method of Pemberton-Kilgore.

Results.

The results of the balance experiments are shown graphically in Figs. 1 to 6. The most striking observation shown is the fact

that in seventeen lactation periods on the various diets all show that the animals were in negative calcium balance. In sixteen periods the phosphorus balances were also negative and in only one period was the animal in phosphorus equilibrium. All but two changed from negative to positive calcium and phosphorus balances following removal of the young.

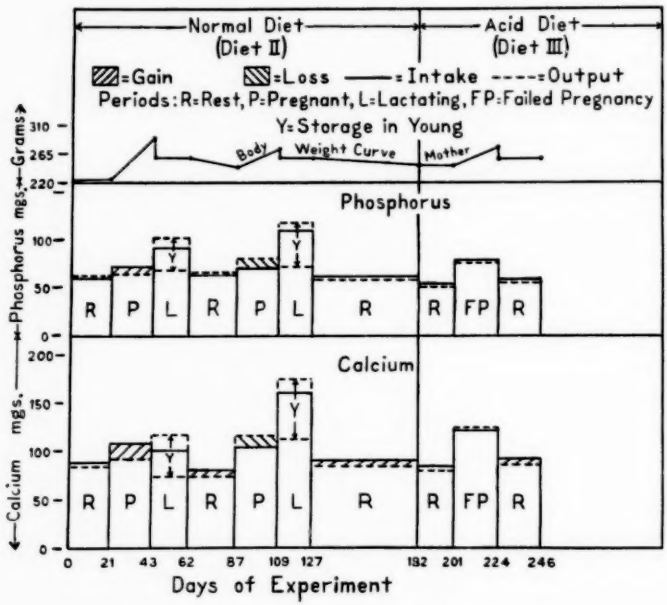

FIG. 1. Balance chart for Rat B8664.

A second observation of note is that during twenty-seven pregnancies on the different diets, in only four was there failure to store calcium and in only three was there a negative balance of phosphorus.

Within the range of acidity employed in Diet III, no marked difference in calcium and phosphorus balance over the normal diet is shown. The animals showed a remarkable capacity to thrive and reproduce normal young on this diet. This is likewise true of alkaline Diet IV. It will be noted, however, that both animals

maintained on Diet IV failed to lactate the young after the second pregnancy. The first litter in each instance was reared. The second pregnancy was normal and full weight young were born to each mother. In both cases the young were not nourished and died of starvation. It is not possible at this time to ascribe the phenomenon to the diet since the number of animals is not sufficient for statistical purposes. It is noteworthy, however, that

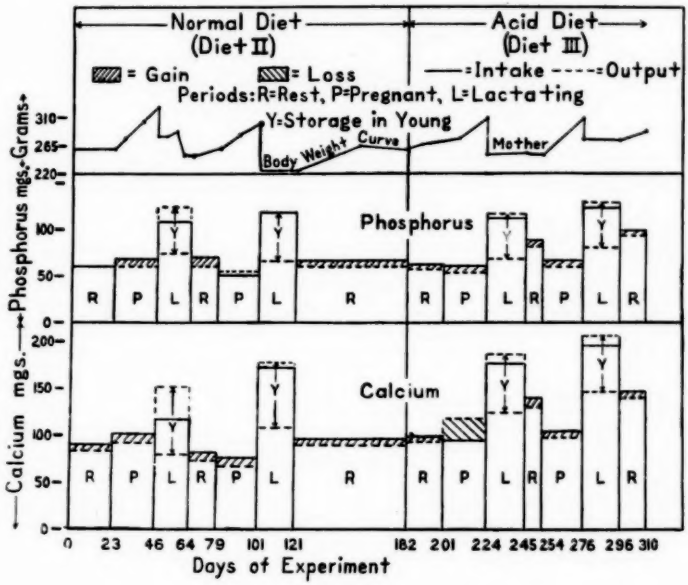

FIG. 2. Balance chart for Rat BH8307.

when after a short rest period these two females were changed to the normal diet[2] of the stock colony, they produced and reared normal young. Further experiments to elucidate this question are in progress.

In Fig. 7 are given the results of a growth experiment in which it was desired to determine the rate of assimilation of the calcium

[2]The stock diet consisted of yellow corn 76, linseed oil meal 16, casein 5, alfalfa meal 2, sodium chloride 0.5, calcium carbonate 0.5.

and of the phosphorus in young rats maintained on Diet III and Diet IV respectively. Two young from Rat 10 were placed in a single metabolism cage at the conclusion of the lactation period and calcium and phosphorus balances were carried out for 7 or 8 day periods until six such periods were completed. A similar experiment was carried out on two young from Rat W19 with the alkaline diet. The animals which were maintained on the alkaline

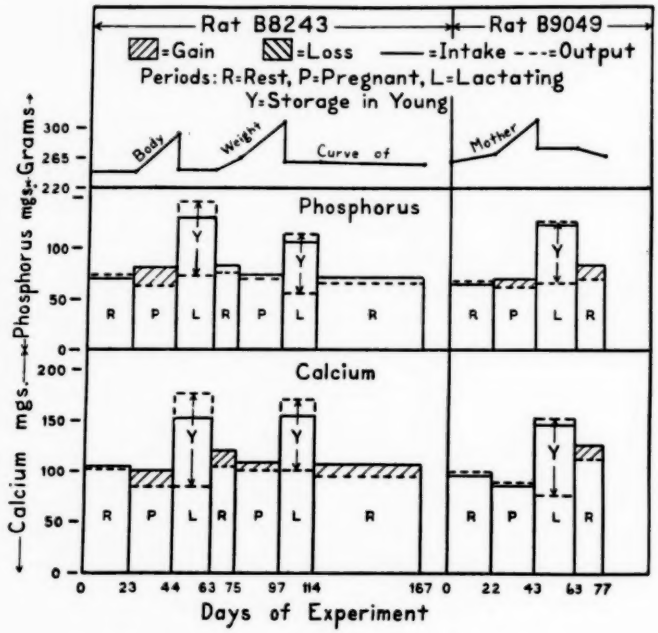

FIG. 3. Balance chart for rats on normal diet (Diet II).

diet grew at a slightly greater rate. The calcium and phosphorus storage is normal in both cases. The figures show the extraordinary ability of young rats to assimilate and retain both calcium and phosphorus irrespective of the reaction (within the limits studied) of the diet.

In Figs. 8 and 9 are given the results of a study of the calcium and phosphorus metabolism of rats maintained on a purified diet

containing all of the essential factors except vitamin E. Females which had been reared on the Evans and Burr (10) Diet 232[3] and which had undergone one resorption of the young were used in these experiments. The animals were maintained on Diet 228 to which yeast was added. After a normal period during which no vitamin E was included in the diet, three animals were given

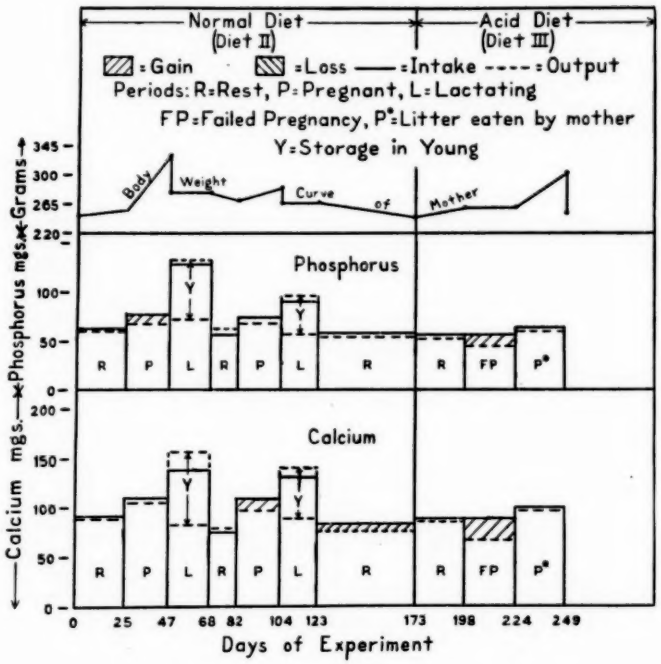

FIG. 4. Balance chart for Rat GHS011.

6 drops of wheat germ oil daily while the other three were kept as controls without this vitamin. No effect on the calcium and phosphorus balances was observed following administration of vitamin E. The animals were then mated. Those which received vitamin E gave birth to young while the controls resorbed.

[3] Diet 232 consisted of casein 32 gm., corn-starch 40 gm., lard 22 gm., cod liver oil 2 gm., Salt Mixture 185 4 gm., and yeast 0.6 gm. daily.

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TABLE II.

Calcium Balance during Pregnancy.

Rat No.	BH9393	W9337
Weight at littering, *gm.*	230	286
" of fetus, *gm.*	28.1	28.7
" " placenta and uterus, *gm.*	7.7	11.3
Food consumption, *gm.*	297	343
Total Ca in food, *mg.*	2432	2812
" " excreted, *mg.*	2085	2425
Ca balance, *mg.*	+347	+387
" found in fetus, *mg.*	65	63
" in uterus and placenta, *mg.*	3	3
Excess Ca stored, *mg.*	279	321

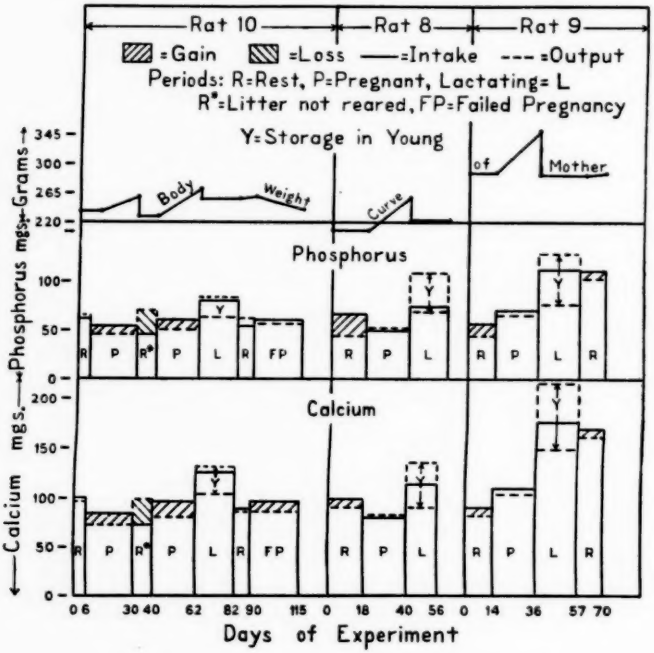

FIG. 5. Balance chart for rats on acid diet (Diet III).

Rat G2967 following mating showed a large retention of both calcium and phosphorus during the first 7 days of gestation. This could not have been stored in the embryos since implantation does not occur until about the 6th day of gestation. In all of the

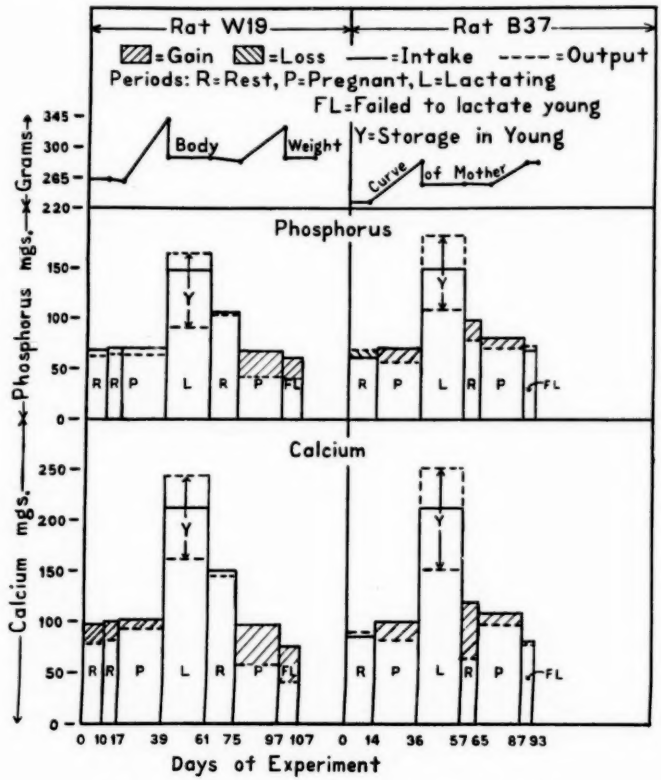

FIG. 6. Balance chart for rats on Diet IV ($NaHCO_3$).

animals including those which resorbed there was a positive balance of both calcium and phosphorus following mating.

Our experiments have indicated a storage of calcium and phosphorus during pregnancy. Table II shows the distribution of

these stored elements. The data were obtained from two female rats which were placed on a balance experiment. Just before the birth of the young the mothers were killed and the uteri,

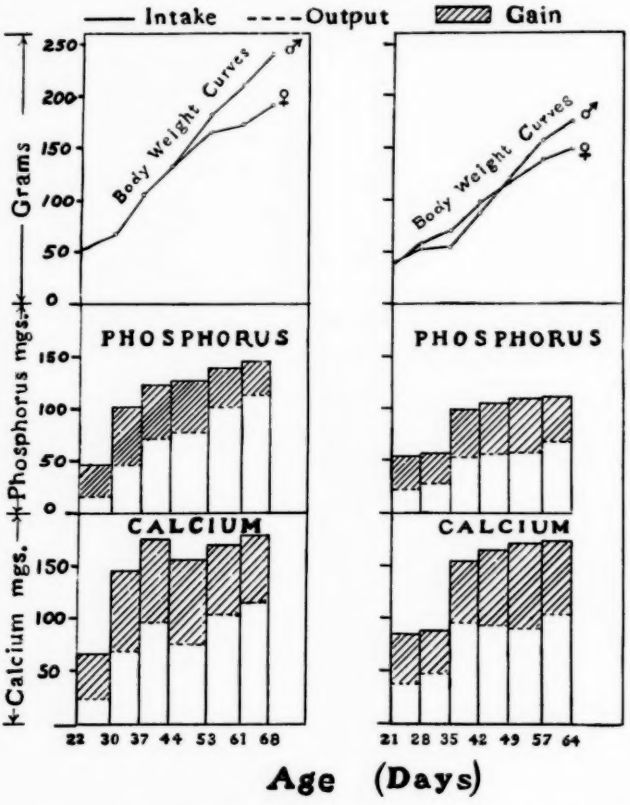

FIG. 7. Balance chart for rats on an acid and an alkaline diet. The results shown on the left are those for two young from Rat W19 fed Diet IV ($NaHCO_3$); on the right, for two young from Rat 10 fed Diet III (HCl).

fetuses, and placentæ were removed and separated. It will be noted that the storage of calcium is four to five times that which is contained in the fetus plus the placenta and uterus.

No direct correlation was found between the size of the litters reared and the magnitude of the negative balance of the mother during the lactation period. In general, as might be expected, the largest losses of calcium were associated with the largest litters. Since every lactation period resulted in a loss of calcium

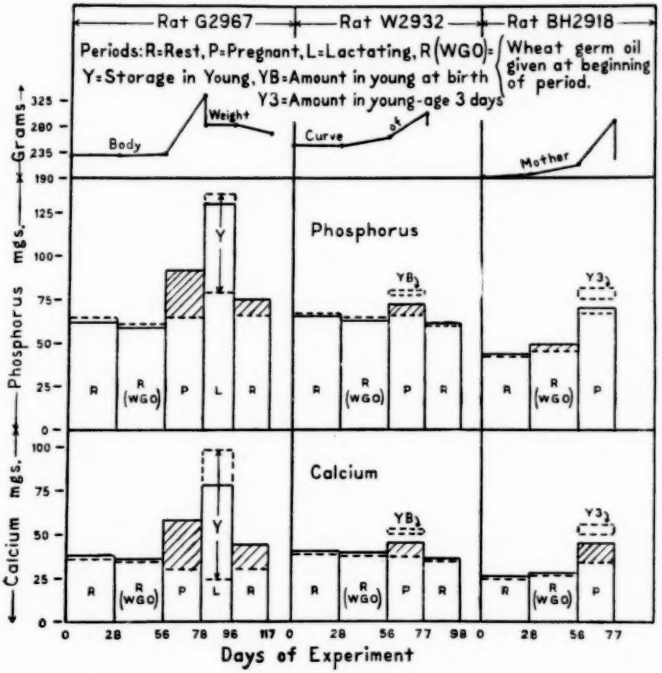

FIG. 8. Balance chart for rats on Diet 228 + 0.7 gm. of yeast daily. Gain, loss, intake, and output are indicated in the same manner as in the other figures.

it is of interest to note the extent of these losses from the body of the mother. The total calcium content of an adult female rat is about 3 gm. and the phosphorus content is about 2 gm. We have calculated that during a heavy lactation period the rat may lose 20 per cent of her total supply of calcium and phosphorus. Since

only about 1 per cent of the body calcium is not contained in the bones, the loss must be carried by the skeleton. Bauer, Aub, and

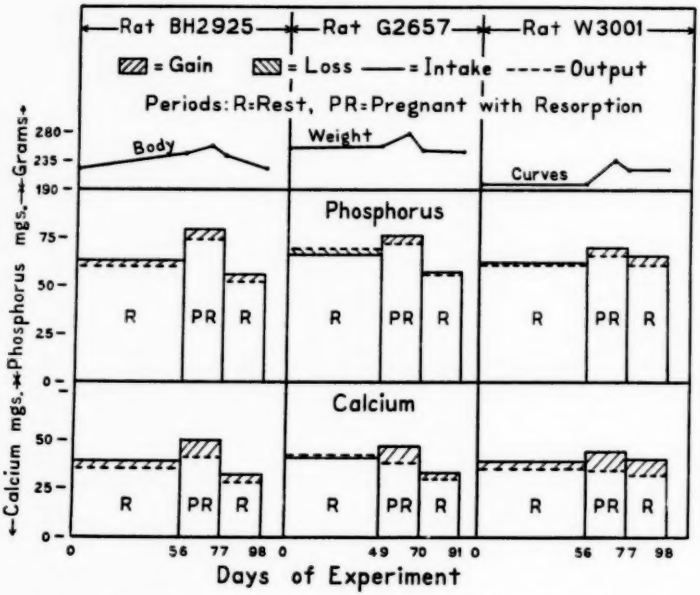

FIG. 9. Balance chart for rats on Diet 228 + 0.7 gm. of yeast (vitamin E-free).

TABLE III.

Analyses of 166 New Born Rats.

	Moisture.	Ca	P
	per cent	*per cent*	*per cent*
Maximum	86.7	0.289	0.305
Minimum	85.0	0.230	0.277
Average	85.8	0.260	0.295

Albright (12) have concluded that the trabeculæ of the long bones serve as a storehouse of conveniently available calcium.

While it appears from these experiments that within the range of reaction used there is no marked effect of acidity upon calcium and phosphorus absorption during pregnancy and lactation in the rat, the statement cannot be taken as absolute, since from a statistical point of view a sufficient number of animals may not have been employed. The inability of the rat to absorb sufficient calcium from the diet to meet its lactation requirements is striking. Whether this is a problem of ultimate permeability of the intestinal wall or whether factors can operate to increase the calcium transport through the intestines are questions which are not answered by these experiments. It is of interest to note the constancy of calcium and phosphorus in the bodies of young rats at birth. In Table III are summarized the results of analyses of 166 new born rats. They represent twenty-five litters from mothers on various natural and pure food diets. Sherman and MacLeod (13) and Sherman and Quinn (14) give the following figures for newly born rats: calcium 0.25 per cent and phosphorus 0.34 per cent. The magnitude of our values is in approximate agreement with these figures.

The analytical data relating to the experiments described in this paper are on file in the University of California library.

SUMMARY.

1. Calcium and phosphorus balances in the rat have been carried out through continuous periods of rest, pregnancy, and lactation. The reaction of the natural food diets was varied from alkaline to acid in different experiments.

2. During twenty-seven completed pregnancy periods, on the various diets, only four failed to show a positive balance of calcium and only three failed to store phosphorus. In most cases the positive balance was in excess of that estimated to be contained in the young at birth.

3. In seventeen lactation periods, all showed a negative balance of calcium and all but one a loss of phosphorus. The losses, for full sized litters were as great as 20 per cent of the entire body content of these elements in the mother.

4. Within the range of acidity employed, no significant difference could be found between the diets, except that the second

litters from the rats on the diet containing sodium bicarbonate were not nourished by the mother. This was due to failure to lactate.

5. Young rats from mothers which had been maintained on acid Diet III and alkaline Diet IV respectively, were kept for some time on these respective diets. No difference in storage of calcium and phosphorus was noted.

6. Females reared on a vitamin E-free diet stored calcium and phosphorus during the period of pregnancy in which resorption of the fetus took place.

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THE COMPOSITION OF PANCREATIC JUICE AND BLOOD SERUM AS INFLUENCED BY THE INJECTION OF ACID AND BASE.*

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INTRODUCTION.

The composition of the external secretions of various glands of the body is actively controlled by the glands themselves. These secretions, though composed of materials supplied by the same circulating blood serum, differ widely in their composition and reaction. We have, for example, nearly neutral saliva, acid gastric juice, alkaline bile and pancreatic juice; and we may add to this list urine of widely varying composition and reaction. This suggests (*a*) that the gland acts as a semipermeable membrane of unknown complexity and (*b*) that within the gland chemical reactions between diffusible substances and non-diffusible materials probably occur.

It was thought that some information might be obtained concerning the formation of a fluid secreted by a gland by studying the changes in the composition of the secreted fluid brought about by experimental variations in the composition of the blood serum during the period of secretion. In this paper data are presented to show the effect of intravenous injections of sodium carbonate and hydrochloric acid on the hydrogen ion, carbon dioxide, and chloride concentrations of both pancreatic juice and serum.

* This and the following paper constitute a thesis submitted to the Graduate School of the University of Pennsylvania in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The investigation was carried out under the direction of Dr. D. Wright Wilson.

† Henry Strong Denison Medical Foundation Fellow, 1928–29.

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Data on the changes in the concentrations of these same constituents in pancreatic juice with variations in glandular activity are also given.

The alkalinity of pancreatic juice as reported by previous workers varies over a wide range. Bayliss and Starling (1) reported pancreatic juice with an alkalinity equivalent to a 0.1 N sodium carbonate solution. Other workers, DeZilwa (2), Morel and Terroine (3), and Mellanby (4), found titratable alkalinities varying from 0.05 N to 0.16 N. pH values for pancreatic juice show as wide a variation: Foa, 9.79 to 10.21 (5); Auerbach and Pick, 7.3 to 8.7 (6); Archangelskii, 8.41 to 8.53 (7); Kestner and Plaut, 7.34 (8); Carnot and Gruzewska, 8.71 to 8.98 (9); Dubois and Polonovski, 7.8 to 8.7 (10); Still and Barlow, 7.85 to 8.30 (11). Czubalski (12) showed that the alkalinity of pancreatic juice varied with the rate of secretion, obtaining pH values ranging from 7.06 on slowly secreted juice to 8.64 on juice more rapidly secreted. Other workers report this same observation (Glaessner (13) and Wohlgemuth (14)). Some of these values were determined electrometrically, though none of the workers state definitely what precautions were used to prevent loss of free carbon dioxide while collecting the juice. More recently Gamble and McIver (15) have shown that the chloride and bicarbonate concentrations of pancreatic juice vary inversely and suggest that the alkalinity is thereby controlled.

Experimental Methods.

Large dogs (15 to 20 kilos) were used in order to obtain pancreatic ducts that could be easily cannulated and to insure a large flow of juice. An animal that had not been fed for the previous 24 hours was anesthetized by an intraperitoneal injection of amytal, a right rectus incision was made, and the duodenum and adjoining pancreas delivered into the wound, care being taken to handle the pancreas as little as possible. A glass cannula was then tied in the major pancreatic duct and at least one accessory duct ligated. In order to prevent kinking of the duct the cannula was held in position by suturing it to the intestine. Glass tubing was connected to the cannula by means of a short piece of rubber tubing. The duodenum was returned to the abdominal cavity and the incision closed around the glass tubing.

Since there was not a normal flow of juice immediately after the operation, secretin was injected into the jugular vein. The secretin was prepared by the method of Cowgill and Mendel (16), which was the most convenient and yielded the most active preparation of any method tried. Juice so obtained was clear and

colorless. After the juice had filled the glass tubing connected to the cannula, a tonometer filled with mercury was connected and the juice collected under a slight negative pressure. This procedure was found necessary when the hydrogen ion concentration was to be determined. Pancreatic juice is so poorly buffered that when collections are made under oil sufficient loss of free carbon dioxide occurs to cause appreciable pH changes. Some samples were collected under oil in graduated 15 cc. centrifuge tubes, to enable the rate of secretion to be determined. Samples collected in this manner are designated in the tables.

Since the rate of flow of pancreatic juice affects its alkalinity, the rate of secretion was kept as uniform as possible by injecting secretin at regular intervals, usually every 15 minutes. The first sample obtained was generally collected under oil, and considered to be a mixture of the preformed juice in the gland (capacity of ducts in the cat 1 to 1.5 cc. (17)), and the juice called forth by the secretin. A second sample, presumably true secretin juice, was then collected in a tonometer. An initial sample of blood was taken from the jugular vein, then an intravenous injection of a solution of acid or base was made followed by the drawing of a second blood sample. Two more samples of pancreatic juice were collected in tonometers and then a third blood sample was taken. Sometimes further collections were made. All blood samples were collected under oil, which was replaced by paraffin before centrifuging. The serum was drawn off into a tonometer. The animal was under anesthesia throughout the experiment and sacrificed at the end.

Analytical Methods.

Total carbon dioxide was determined on juice and serum by the method of Van Slyke and Stadie (18). Carbon dioxide tension of both fluids, reported as H_2CO_3, was calculated from the total carbon dioxide and pH values by means of the following equation which is a combination of equations developed by Van Slyke.

$$\text{mM } H_2CO_3 = \frac{\text{mM total } CO_2}{10^{pH - pK'} + 1}$$

The value of 6.1 for pK′ of serum, determined by Hastings, Sendroy, and Van Slyke (19) was used. Since the ionic activity of

pancreatic juice is probably similar to that of serum, it was assumed that this value for pK′ could also be used for pancreatic juice. Bound carbon dioxide, reported as HCO_3, was taken as the difference between total and free carbon dioxide. When no pH value was obtained, an average value of one mM of free carbonic acid was assumed for both serum and pancreatic juice, and bound carbon dioxide calculated accordingly. Since the free carbon dioxide is but a small fraction of the total, especially in the juice, any error in such calculation will cause but a negligible error in calculating the bound carbon dioxide concentration.

Chloride was determined by the method of Van Slyke as modified by Wilson and Ball (20).

Sodium was determined by the method of Kramer and Gittleman as modified by Kerr (21). Serum values were obtained on ashed samples, since direct determinations were found to yield unreliable results as reported by Kerr. Pancreatic juice was analyzed without ashing, since ashed and unashed samples gave identical results. Several samples of pancreatic juice, analyzed by both the micro and the macro chloroplatinate methods, gave checks within the experimental error.

The hydrogen ion concentrations of serum and of pancreatic juice were determined by means of the quinhydrone electrode[1] and colorimetrically at 38°. All samples were run within 6 hours of collection; no significant change in pH occurs in tonometer samples that have been allowed to stand this long. The quinhydrone method was shown to yield results, with dog serum, that agreed favorably with the hydrogen electrode determinations. Samples of pancreatic juice collected in tonometers run by both methods gave pH values with the quinhydrone electrode of (1) 8.16 and (2) 7.93, and with the hydrogen electrode of (1) 8.14 and (2) 7.92. Colorimetric determinations on both fluids were run by the Hastings and Sendroy (22) modification of the Cullen method. Colorimetric and quinhydrone pH values for pancreatic juice agreed within experimental errors. The two methods showed variations with serum of a magnitude similar to those reported by Johnston (23) for the hydrogen electrode and the colorimetric methods. pH values reported here are quinhydrone determinations unless otherwise stated.

[1] I wish to thank Mr. E. P. Laug for making these determinations.

Results.

Table I shows the influence of secretion rate on the juice composition. The more rapidly a juice was secreted the higher were the pH value and bound carbon dioxide concentration and the lower the chloride. Thus the concentrations of chloride and bicarbonate varied inversely, their sum being more or less constant regardless of the rate of secretion. A slowly secreted juice had a

TABLE I.

Influence of Secretion Rate on Juice Composition.

Dog No.	Juice No.*	Secretion rate.	mM per liter.			$\frac{HCO_3}{Cl}$	pH†
			HCO_3	Cl	$HCO_3 + Cl$		
		cc. per min.					
27	2	0.29	93	47	140	2.0	8.12
	A	0.04	34	108	142	0.3	7.65
26	4 A	0.65	134	26	160	5.1	8.15
	5 A	0.12	86	66	152	1.3	8.05
23	2	0.19	120	36	156	3.3	
	3	0.03	56	79	135	0.7	
21	2	0.34	116	38	154	3.0	
	3	0.05	63	84	147	0.8	
20	2	0.42	126				
	1	0.01	46				

* All collected under oil.

† Colorimetric.

ratio of the concentrations of bicarbonate to chloride approaching that found in blood serum, namely 0.2, while in a rapidly secreted juice the magnitudes of the concentrations were reversed and a ratio of 3.3 obtained. This effect of secretion rate on juice composition was most noticeable when there was a large variation in the rate. Juices secreted rapidly but varying as much as 50 per cent in rate did not seem to show appreciable variations in composition.

In Tables II and III are recorded the effects of the intravenous injection of 0.5 N HCl in 0.9 per cent saline solution on the com-

position of blood serum and pancreatic juice. Acid injections were made very slowly and were accompanied by marked hyperpnea; no effect on the flow of pancreatic juice was observed.

Table II shows the results of an experiment in which the injection of acid caused a fall of 0.1 of a pH unit in the blood serum,

TABLE II.

Injection of Hydrochloric Acid.

Dog 29, weight 17.6 kilos.

Sample.	Time.	Amount.	Rate.	pH	mM per liter.			
					H_2CO_3	HCO_3	Cl	$HCO_3 + Cl$
		cc.	*cc. per min.*					
Juice 1.*	10.35–10.40 a.m.	2.7	0.54			102	47	149
" 2.	10.40–11.15 a.m.	14	0.40	8.14	1.2	132	24	156
Serum 1.	11.15 "	40†		7.22	1.6	23	113	136
Injection.	11.33 " – 12.50 p.m.	72		0.5 N HCl in 0.9 per cent NaCl intravenously.				
Serum 2.	12.57 "	30†		7.12	1.6	16	119	135
Juice 3.	12.52–1.25 p.m.	12	0.36	8.17	1.0	122	31	153
" 4.	1.30–2.00 p.m.	20	0.66	8.18	1.0	121	30	151
Serum 3.	1.58 "	40†		7.11	1.7	16	118	134
Hemorrhage.	2.00 "	200†						
Juice 5.*	2.00–2.30 p.m.	12.5	0.42			120	33	153

* Collected under oil.

† Amount of whole blood.

while the pancreatic juice showed a slight indication of an alkaline swing due mainly to a decrease in CO_2 tension. The bicarbonate and chloride contents of serum and pancreatic juice fell or rose an equivalent amount. The free CO_2 concentration of the blood serum was higher than that of the juice from the active gland. A light hemorrhage caused no change in the response of the gland to secretin or in the composition of the juice.

It will be observed that the initial blood serum is more acid

than normal. This has been found to be the case in all the dogs in this series of experiments, the quinhydrone results being con-

TABLE III.

Injection of Hydrochloric Acid.

Dog 26, weight 22.9 kilos.

Sample.	Time.	Amount.	Rate.	pH	mM per liter.			
					H_2CO_3	HCO_3	Cl	HCO_3 + Cl
		cc.	cc. per min.					
Juice 1 A.*	10.52–11.02 a.m.	11.5	1.15			134	25	159
" 1 B.*	10.52–11.08 a.m.	5.6	0.35			125	30	155
Serum 1.	11.15 "	40†		7.22	1.7	23	110	133
Juice 2 A.	11.03–11.40 a.m.	18	0.49	8.12	1.3	137	21	158
" 2 B.*	11.08–11.35 a.m.	7.2	0.26	8.12	1.3	133	22	155
Injection.	11.45 "–12.28 p.m.	50		0.5 N HCl in 0.9 per cent NaCl intravenously.				
Serum 2.	12.34 "	50†		7.31	1.2	20	114	134
Juice 3 A.	12.30–12.55 p.m.	18	0.72	8.14	1.2	129	29	158
" 3 B.*	12.30–12.58 p.m.	7	0.25	8.14	1.2	135	31	166
" 4 A.*	12.55–1.15 p.m.	13	0.65	8.10	1.3	134	26	160
Serum 3.	1.10 "	40†		7.22	1.6	20	113	133
Juice 4 B.*	12.58–1.30 p.m.	8.5	0.26			137	27	164
" 5 A.*	1.15–2.00 p.m.	5.4	0.12			86	66	152
Hemorrhage.	6.15 "	500†						
Juice 6 A.*	6.15–6.33 p.m.	7.4	0.41			103	53	156
Serum 4.	6.30 "	40†				19	112	131

* Collected under oil.

† Amount of whole blood.

firmed by hydrogen electrode determinations. The cause of this acid condition was not investigated. A contributing factor may have been the use of amytal, as a marked decrease in respiratory

rate can be observed when the anesthetic has taken effect. However, Hines, Boyd, and Leese (24) have reported that amytal does not affect the pH of blood serum.

The results given in Table III were obtained on a dog in which two ducts from different parts of the pancreas were cannulated. Juices from these were labeled A and B. Only slight differences in the composition of the juices were noted. The acid injection

TABLE IV.

Injection of Sodium Carbonate.

Dog 32, weight 12.4 kilos.

Sample.	Time.	Amount.	Rate.	pH	mM per liter.				
					H_2CO_3	HCO_3	Cl	$HCO_3 + Cl$	Na
		cc.	*cc. per min.*						
Juice 1.*	9.01–9.14 a.m.	3.8	0.29			100	49	149	
" 2.	9.14–9.50 a.m.	10	0.28	8.13	1.1	120	33	153	157
Serum 1.	10.10 "	35†		7.27	1.4	20	110	130	133
Injection.	10.30–11.55 a.m.	100		10 per cent Na_2CO_3 intravenously.					
Serum 2.	11.35 "	40†		7.38	1.7	33	105	138	154
Juice 3.	11.30 "–12.15 p.m.	12	0.27	8.15	1.2	135	33	168	181
" 4.	12.15–1.05 p.m.	16	0.32	8.24	1.0	133	37	170	181
Serum 3.	12.55 "	40†		7.36	1.8	33	108	141	155

* Collected under oil.

† Amount of whole blood.

in this experiment had little effect on the bound carbon dioxide and the serum actually became alkaline probably due to overventilation. The composition of the pancreatic juice showed little significant change throughout the experiment. Hemorrhage again produced no noticeable effects.

In Tables IV, V, and VI are tabulated the effects of the intravenous injection of a 10 per cent sodium carbonate solution on the

composition of serum and pancreatic juice. No effect on the flow of juice was observed. In the experiment recorded in Table IV the injection of sodium carbonate caused the serum to become 0.1 pH more alkaline while the pancreatic juice showed a delayed rise of the same magnitude. The bicarbonate concentration of serum and pancreatic juice increased similarly after the injection, chloride values remaining more or less unchanged. Juice and

TABLE V.

Injection of Sodium Carbonate.

Dog 28, weight 17.6 kilos.

Sample.	Time.	Amount.	Rate.	pH	mM per liter.				
					H_2CO_3	HCO_3	Cl	$HCO_3 + Cl$	Na
		cc.	*cc. per min.*						
Juice 1.	10.12–10.47 a.m.	10	0.29	8.03	1.5	122	33	155	158
Serum 1.	10.21 "	40†		7.17	2.0	24	106	130	141
Injection.	10.52 "–12.08 p.m.	95		10 per cent Na_2CO_3 intravenously.					
Serum 2.	12.11 "	40†		7.45	1.9	41	106	147	154
Juice 2.	12.08–12.38 p.m.	16	0.53	8.01	1.7	138	34	172	175
" 3.	12.38–1.10 p.m.	16	0.50	8.09	1.3	128	37	165	174
Serum 3.	1.13 "	40†		7.36	1.8	33	108	141	151
Juice 4.*	1.10–1.45 p.m.	12.5	0.36	8.08	1.3	120	47	167	173

* Collected under oil.

† Amount of whole blood.

serum carbon dioxide tensions increased, the latter markedly, after the injection. The increase in sodium concentration of the serum was reflected quantitatively in the juice. This will be discussed in another paper.

Table V shows the sodium carbonate to have caused an alkaline swing of nearly 0.3 of a pH unit in the serum while a delayed rise of only 0.06 of a pH unit occurred in the juice. Values for bi-

carbonate and chloride concentrations were similar to those in Table IV. Serum carbon dioxide tension was lowered after injection while that of the pancreatic juice again increased.

TABLE VI.

Injection of Sodium Carbonate.

Dog 27, weight 12.1 kilos.

Sample.	Time.	Amount.	Rate.	pH	mM per liter.				
					H_2CO_3	HCO_3	Cl	$HCO_3 + Cl$	Na
		cc.	cc. per min.						
Juice 1.*	8.17–8.35 a.m.	5.6	0.31			95	51	146	
Serum 1.	8.33 "	40†		7.27	1.4	20	107	127	135
Juice 2.	8.35–9.10 a.m.	10	0.29	8.20	0.8	93	47	140	149
Injection.	10.11–10.40 a.m.	12		10 per cent Na_2CO_3 intravenously.					
Serum 2.	10.43 "	40†		7.35	1.3	23	106	129	
Juice 3.	10.42–11.12 a.m.	10	0.33	8.20	0.8	102	48	150	151
" 4.*	11.12–11.43 a.m.	5.5	0.18	8.19	0.8	101	46	147	
" 5.*	11.47 " –12.15 p.m.	4.2	0.13	8.10	1.0	96	51	147	
Hemorrhage.	12.27 "	230†							
Injection.	12.29–12.43 p.m.	23		10 per cent Na_2CO_3 intravenously.					
Juice 6.*	12.44–1.15 p.m.	4.8	0.15	8.02	1.0	85	68	153	159
Injection.	1.00–1.11 p.m.	35		10 per cent Na_2CO_3 intravenously.					
Serum 4.	1.23 "	40†		7.59	1.1	34	103	137	153
Juice 7.	1.16–1.55 p.m.	7.3	0.19	8.02	1.3	109	49	158	171

* Collected under oil.

† Amount of whole blood.

The results given in Table VI are those of a longer experiment. Three injections of sodium carbonate were made causing the pH and the bicarbonate concentration of the serum to increase.

The pancreatic juice also showed at first a similar increase of bicarbonate content with no change in pH. In later juices decreased pH values and bicarbonate concentrations with increased carbon dioxide tensions appeared in spite of the alkaline swing of the serum. It is possible that this was partly the result of a change in rate of flow.

It would seem that the main factor controlling the alkalinity of pancreatic juice is the rate of flow. The more rapid the rate of secretion, the higher the alkalinity of the juice. Intravenous injection of acid causing a lowered bicarbonate concentration of the serum and an acid swing, had little effect on pH values of pancreatic juice, though the bicarbonate content fell in an amount similar to that of the serum. Intravenous injections of sodium carbonate, causing an increased bicarbonate concentration and an alkaline pH swing of the serum, had a similar though less effect on the composition of pancreatic juice. The bicarbonate content increased immediately and in amount similar to that of the serum; pH values showed a delayed rise of smaller magnitude due mainly to the independence of the serum and pancreatic juice carbon dioxide tensions.

In other experiments in which acid salts (NaH_2PO_4) and lactic acid were injected, no significant pH changes in the serum or pancreatic juice were observed and the data are not reported; the injection of the lactic acid caused marked hemolysis.

DISCUSSION.

Though previous workers have claimed that pancreatic juice contains carbonate, values for bound carbon dioxide reported here are expressed as bicarbonate. Justification for this is seen if the ratio of carbonate to bicarbonate in a solution is calculated at a pH of 8.24, the highest value obtained in these experiments. Using the value of 10.22 for pK_2' of carbonic acid given by Michaelis (25):

$$\text{Log}\ \frac{\text{salt}}{\text{acid}} = \text{pH} - \text{pK}_2'$$

$$= 8.24 - 10.22$$

$$\frac{\text{Salt}}{\text{Acid}} = \frac{1}{100}$$

In a juice with a pH of 8.24 about 1 per cent of the bound carbon dioxide is present as carbonate.

Since the juice bicarbonate is remarkably independent of the serum bicarbonate, it would seem that the serum is not the direct source of the juice bicarbonate. Hammarsten and Jorpes (26) have recently reported that prolonged secretion of pancreatic juice does not affect the alkaline reserve of the blood but does cause an acid swing in extracts made of the gland itself. They believe their results show that the sodium bicarbonate of the juice is formed by the action of the carbonic acid produced by the active gland on the alkali compounds, possibly salts of nucleic acids, of the pancreatic cells. The hydrogen ion is retained by the non-diffusible compounds and the sodium bicarbonate passes into the juice. The data presented in the present paper show that the alkalinity of the pancreatic juice increases with increased glandular activity, and that the carbon dioxide tension of the juice from the active gland is usually lower than the venous blood of the animal. These results would seem to support Hammarsten and Jorpes' conclusion that the carbon dioxide produced by the working gland is used to form a large part of the bound carbon dioxide of the juice it produces. However, it would seem from experiments reported in the following paper that the blood serum is the immediate source of the bases of the juice rather than alkali compounds of the gland itself. At any rate the pancreas appears to function so as to permit the free passage of base and the retention of hydrogen ions; the concentration of chloride and bicarbonate, and hence the alkalinity of the juice, varying with the gland's activity. This suggests the contrasting action of the stomach which appears to permit the free passage of chloride, and varies the concentration of hydrogen ion and base with changes in gland activity (McCann (27)).

On the other hand, Woinar (28) drew the conclusion that the total carbon dioxide content of pancreatic juice depends on the alkaline reserve of the blood serum and *vice versa*. He reported that continuous secretion of pancreatic juice causes a fall in serum alkaline reserve and a marked decrease in juice alkalinity which is, however, accompanied by a decreased rate of flow. Intravenous injection of sodium carbonate after prolonged secretion raises the juice alkalinity to above its original value as well as its rate of secretion. If the serum alkaline reserve is artificially lowered by

intravenous injections of acid, the bicarbonate concentration of the juice decreases as much as 50 per cent, the rate of flow also decreasing markedly. The data is the present paper indicate that Woinar probably failed to realize the importance of the rate of flow in influencing juice composition and for this reason was led to draw the erroneous conclusion that compositions of juice and serum run parallel.

Since the free carbon dioxide content of pancreatic juice is relatively constant and the rate of flow determines its bound carbon dioxide concentration and hence its alkalinity, it is of interest to inquire what the highest possible alkalinity of pancreatic juice is likely to be. The maximum rate of secretion obtained in these experiments is about 60 cc. per hour. This value compares favorably with the maxima obtained on dogs by other workers. Pawlow (29) reports values of 70 to 80 cc. an hour for large dogs. Elman and McCaughan (30) obtained a maximum of 500 cc. daily. This would seem to indicate that with the rapid rate of flow, pH values reported here are probably nearly maximal. However, it might be possible with a more rapid flow that all the inorganic cations would exist as bicarbonate ions. Since pancreatic juice is in osmotic balance with serum, as will be shown in the following paper, an inorganic cation concentration much greater than 150 mM is impossible in the normal animal. It might be argued that a very active gland would secrete carbonate instead of bicarbonate. If this occurred the total carbon dioxide content of the juice would be halved, for the constant amount of base in the juice could bind only half as much carbon dioxide in the form of carbonate as it would in the form of bicarbonate. In view of the fact that the bound carbon dioxide always increases as the rate of flow increases, there seems to be no tendency for carbonate to replace bicarbonate.

To calculate the maximum pH possible, we may assume a constant free carbon dioxide concentration of 1 millimol and a maximum bicarbonate of 150 millimols per liter.

$$\begin{aligned} \text{pH} &= \text{pK}' + \log \frac{\text{salt}}{\text{acid}} \\ &= 6.14 + \log \frac{150}{1} \\ &= 8.32 \end{aligned}$$

If these assumptions are correct, the most alkaline juice obtainable has a pH of about 8.3. It would seem that values more alkaline than this, as reported by some workers, are due to loss of free carbon dioxide during collection. Since pancreatic juice is poorly buffered, the escape of free carbon dioxide may cause a marked alkaline swing. Even when juice is collected under oil, increase in alkalinity on standing is noticeable. For example, one sample collected under oil when first analyzed gave a pH of 8.10; after standing 3 hours a value of 8.16 was obtained.

On the other hand, juices secreted very slowly may have low pH values. Values similar to those of blood serum will be reported in permanent fistula dogs by Johnston and Ball (31). These juices have bicarbonate and chloride concentrations similar to blood serum.

It is of interest to compare the results obtained by other workers in similar experiments but on different body fluids. Carnot and Gruzewska (32) found that intravenous injections of sodium bicarbonate caused bile to become much more alkaline, the bicarbonate concentration of the bile increasing considerably. Gesell (33) showed that intravenous injections of sodium bicarbonate increased the alkalinity of lymph and blood about equally. Gesell and Hertzman (34) reported that sodium carbonate intravenously injected causes an increased alkalinity of cerebrospinal fluid while injections of sodium bicarbonate caused an acid swing in the fluid. They claim this difference in action of the two salts is due to the non-permeability of the base. Though bicarbonate injections were not made in the present study of pancreatic juice, it is not likely that a case analogous to cerebrospinal fluid would be found since the pancreatic cell seems to be freely permeable to sodium. Hug and Marenzi (35) found that intravenous injection of acid caused bile to become more alkaline, the bicarbonate content increasing. In a similar study on saliva, a slight increase in alkalinity was noted but a decrease occurred in the bicarbonate content. These results are similar to the effects of acid injection on the composition of pancreatic juice.

SUMMARY.

1. A study of the alkalinity of pancreatic juice and its relation to serum composition has been made.

2. The pH of the juice varies with its rate of flow. Juices secreted rapidly are the more alkaline, containing large amounts of bicarbonate and small amounts of chloride. In slowly secreted juices a lower alkalinity is found with large concentrations of chloride and small concentrations of bicarbonate. The sum of the bicarbonate and chloride concentrations is nearly constant regardless of the rate of flow.

3. Intravenous injections of hydrochloric acid cause but little change in the pH of pancreatic juice, regardless of the effect on the serum. Lowered bicarbonate values for serum are manifested quantitatively though not proportionally in the juice.

4. Intravenous injections of sodium carbonate solution, causing an alkaline swing in the serum, produce remarkably little change in the reaction of the juice. However, the increased concentration of bicarbonate and sodium in the serum appears quantitatively in the juice.

5. The possible significance of these findings and their relation to other studies of a similar nature is discussed.

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THE COMPOSITION OF PANCREATIC JUICE AND BLOOD SERUM AS INFLUENCED BY THE INJECTION OF INORGANIC SALTS.

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INTRODUCTION.

In the previous paper it was suggested that information concerning the permeability of the pancreatic tissue to the constituents of the blood serum could best be studied by determining the composition of pancreatic juice and serum during experimental variation in the composition of the latter. In that paper experiments were reported, in which acid and alkaline solutions were injected intravenously and the effect on the composition of the two fluids noted. It was found that the pH of pancreatic juice was practically not influenced by changes in the pH of the blood.

In this paper it is proposed to report further studies on the permeability of the pancreatic tissue to other ions common to both fluids. Solutions of salts containing one or more of the following ions: sodium, potassium, calcium, magnesium, chloride, and phosphate were injected into the blood stream and the composition of the serum and pancreatic juice before and after injection was determined.

Methods.

Experimental procedures for the collection of the pancreatic juice and serum were the same as described in the previous paper except that all samples were collected under oil. All solutions of salts were injected intravenously (jugular) except potassium chloride, which was injected intraarterially (femoral) to lessen its toxic effect.

The sodium, chloride, and bicarbonate concentrations of juice and serum were determined by the same methods described in the previous paper. The inorganic phosphate of both fluids was determined by the method of Fiske and Subbarow (1). The low concentration of phosphate in pancreatic juice made it necessary to add a certain known amount of phosphate before developing the color.

Potassium was determined on ashed samples of pancreatic juice and serum by the method of Kramer and Tisdall as modified by Kerr (2). Unreliable results were obtained on unashed samples of juice and serum, confirming Kerr's findings for serum. Several samples of pancreatic juice run by this method and the macro chloroplatinate procedure gave results that agreed within 5 per cent.

Calcium was determined by the method of Clark and Collip (3) on unashed samples of serum and juice. Ashed and unashed samples of pancreatic juice gave identical results.

Magnesium was determined by the method outlined by Briggs (4) with slight modifications. The addition of the potassium acid phosphate reagent to the filtrate from the calcium determination of pancreatic juice caused a fine precipitate of protein to form. Addition of the concentrated ammonia reagent had no effect on this precipitate. However it was found that if the order of addition of these two reagents was reversed, the ammonia being added first, then no precipitate occurred upon addition of the potassium acid phosphate. The order of addition of these reagents to the serum filtrate was immaterial. The amount of phosphate in the precipitate obtained was determined by the method of Fiske and Subbarow. Since the concentration of magnesium in the juice is small, standard phosphate solution must be added to the sample in order to give a color depth near that of the standard.

Total nitrogen of pancreatic juice was determined by the micro-Kjeldahl method of Folin and Denis (5). Total solids were determined by drying a measured sample at 105–110° overnight in a platinum crucible and weighing the residue. Freezing points were determined on a few samples of juice and serum by means of the apparatus devised by Stadie and Sunderman[1] (6). Qualitative

[1] I wish to thank Dr. F. W. Sunderman for making these determinations.

tests for sulfates were carried out on samples of juice with negative results.

Results.

In Table I are presented data on the composition of serum and pancreatic juice before and after the intravenous injection of a 10 per cent solution of sodium chloride. The injection had no noticeable effect upon the rate of secretion of the juice. Freezing points of both fluids determined before the injection showed that pancreatic juice and serum have osmotic pressures that are identical. The injection of sodium chloride caused a lowering in the freezing point of both serum and pancreatic juice of about the same magnitude. The fact that Juice 4 did not have a freezing point intermediate between that of Serums 2 and 3, as might be expected, was probably due to the unavoidable lag between the time of secretion of the juice and its collection, caused by the dead space between the secreting cells and the collecting tube. An indication of this lag was also shown by the sodium analysis, for in the first juice collected after the injection the sodium concentration increased 8 millimols, an amount identical to the increase in the serum collected immediately after the injection. The concentrations of potassium in the juice and serum were similar throughout, being unaffected by the injection. It will be noticed that the sums of the principle anion and cation concentrations expressed as mM per liter are somewhat higher in the juice than they are in the corresponding sera. However, if concentrations are expressed as mM per kilo of water, the inorganic compositions of the two fluids are more nearly identical. These relations were found to exist in all experiments and will be discussed in considering the compilation shown in Table VII. The concentrations of bicarbonate and chloride in the juice and serum varied inversely, as was shown in the preceding paper, depending on the rate of secretion of the juice.

Since the total concentration of inorganic salts of pancreatic juice is fairly constant (about 0.9 gm. of ash per 100 cc.), variations in total solids are mainly the result of variations in organic matter, probably protein. Continuous secretion appeared to deplete the gland of its organic materials as shown by the decrease in total solids from Juice 1 to 2. In the period during which Juice 3 was being

slowly secreted, the gland appears to have partly replenished its store of organic matter as shown by the increase of solids in Juice 4, only a small part of which was due to the increased concentration of inorganic salts caused by the injection. In the following juices

TABLE I.

Injection of Sodium Chloride.

Dog 21, weight 16.2 kilos.

Sample.	Time.	Amount.	Δ	Total solids.	N	mM per liter.						mM per kilo water.	
						Na	K	HCO_3	Cl	Na + K	HCO_3 + Cl	Na + K	HCO_3 + Cl
		cc.		*gm. per 100 cc.*	*gm. per 100 cc.*								
Juice 1.	10.38–11.11 a.m.	12		2.15	0.20	151	4.6	113	35	156	148	159	151
Serum 1.	10.56 "	40*	0.561			143	3.3	25		146		159	
Juice 2.	11.32 " – 12.14 p.m.	14.2	0.558	1.74	0.14	151	4.1	117	38	155	155	158	158
" 3.	12.14–1.07 p.m.	2.5			0.20			64	84		148		151
Injection.	12.48–1.05 p.m.	64	Injection of NaCl, 10 per cent intravenously.										
Serum 2.	1.09 "	40*	0.600			151	4.0	23	128	155	151	168	164
Juice 4.	1.07–1.27 p.m.	12.8	0.603	2.33	0.23	159	4.1	130	59	163	189	166	193
Serum 3.	1.28 "	35*	0.582			145	4.3	21	124	149	145	162	158
Juice 5.	1.27–1.50 p.m.	4.0		1.71	0.12		4.4	125	40		165		168
" 6.	4.32–5.35 p.m.	9.5		1.63	0.11	164	5.1	101	61	169	162	172	165
Serum 4.	5.27 "	40*				150	5.3	21	124	155	145	168	158

* Amount of whole blood.

another fall in total solids occurred with continuous secretion. The nitrogen of the juice showed the same variation as the total solids. If the total organic matter is computed by subtracting the total ash from the total solids, the total nitrogen is usually found to be about 16 per cent of the organic matter, as might be

expected if the organic material were protein. However in some juices the nitrogen varied from 11 to 28 per cent of the organic matter.

TABLE II.

Injection of Sodium Chloride.

Dog 19, weight 15.8 kilos.

Sample.	Time.	Amount.	Total solids.	N	mM per liter.					
					Na	K	HCO_3	Cl	Na + K	HCO_3 + Cl
		cc.	gm. per 100 cc.	gm. per 100 cc.						
Juice 1.	11.03–11.21 a.m.	13.7	3.79	0.44	157	4.1	104	54	161	158
" 2.	11.21–11.47 a.m.	13.3	2.24	0.25	157	3.6	107	50	161	157
Serum 1.	11.30 "	20*			138		23	112		135
Juice 3.	11.47 "–12.18 p.m.	16	1.74	0.10	155	3.5	104	57	159	161
Injection.	12.18–†12.50 p.m.	57	Injection of NaCl, 10 per cent intravenously.							
Serum 2.	12.26 "	10*			148			123		
Juice 4.	12.29–12.40 p.m.	8.5	2.64	0.24	170	2.9	113	56	173	169
" 5.	12.40–12.50 p.m.	6.8	2.00	0.13	178	4.2	115	55	182	170
Serum 3.	12.54 "	25*			148		23	128		151
Juice 6.	12.50–1.12 p.m.	11	1.75	0.13	176	3.8	112	58	180	170
" 7.	1.12–2.37 p.m.	3.5		0.18	159		58	104		162
" 8.	2.37–3.06 p.m.	12.9	2.33	0.22	175	4.2	114	53	179	167
Serum 4.	3.12 "	25*			149	5.4	25	121	154	146

* Amount of whole blood.

† 32 cc. in at 12.24 p.m., injection then continued at rate of 1 cc. per min. until 12.50 p.m.

In Table II are presented the results obtained in another experiment where sodium chloride was injected. Here the injection was continued at a slower rate during the collection of Juices 4 and 5.

The results are similar to those obtained in the previous experiment. The increase in chloride concentration of the serum was accompanied not by an increase in chloride concentration of the juice, but by an increase in bicarbonate. Total solids of the juice showed an even more pronounced variation that again seems to depend on the previous state of activity of the gland. In the previous paper where injections of sodium carbonate were made, the

TABLE III.

Injection of Potassium Chloride.

Dog 23, weight 17.1 kilos.

Sample.	Time.	Amount.	Total solids.	mM per liter.					
				K	Na	Cl	HCO_3	Na + K	Cl + HCO_3
		cc.	*gm. per 100 cc.*						
Juice 1.	11.16–11.46 a.m.	5.6		6.0	152	36	121	158	157
" 2.	3.51 p.m.–4.40 p.m.	8.4	1.92	6.1	152	53	105	158	158
Serum 1.	4.15 "	20*		5.2	142	111	22	147	133
Injection.	4.49–5.02 p.m.	20	Injection of KCl, 10 per cent intra-arterially.						
Serum 2.	5.06 "	20*		6.2	141	112	19	147	131
Juice 3.	5.03–5.36 p.m.	7.7	1.94	7.8	151	68	88	159	156
" 4.	5.36–6.28 p.m.	8.7	1.61	6.8	149	69	88	156	157
Serum 3.	6.09 "	20*		10.0	141	114	18	151	132

* Amount of whole blood.

concentration of sodium in both juice and serum increased in the same manner as in these injections of sodium chloride.

In Table III are tabulated the compositions of pancreatic juice and serum before and after the intraarterial injection of 10 per cent potassium chloride. The injection caused no noticeable effect on the secretion of juice. Before the injection, the sodium and potassium concentration per volume of juice showed the usual excess over the serum values. After the injection there was a

delayed rise in the potassium concentration of the serum with an even smaller rise in the juice. This small rise in the juice might be attributed to the fact that the delayed rise in the serum potassium had not yet made itself evident in the juice samples collected.

TABLE IV.

Injection of Potassium Chloride and Sodium Acid Phosphate.

Dog 24, weight 12.2 kilos.

Sample.	Time.	Amount.	Total solids.	mM per liter.						
				K	Na	PO_4	HCO_3	Cl	Na + K	$PO_4 + Cl + HCO_3$
		cc.	gm. per 100 cc.							
Juice 1.	10.40–11.04 a.m.	7.4	2.87	7.0	160	0.18	119	40	167	159
Serum 1.	11.15 "	30*		4.8	146	3.6	22	106	151	132
Juice 2.	11.04–11.36 a.m.	5.6	2.09	5.2	162		126	33	167	159
Injection.	11.40–11.58 a.m.	20	Injection of KCl, 10 per cent intra-arterially.							
Serum 2.	12.08 p.m.	20*		7.3	143	3.4	20	107	150	130
Juice 3.	12.10–12.32 p.m.	7.2	2.32	10.3	163	0.16	113	52	173	165
" 4.	12.32–1.09 p.m.	7.5	1.89	12.2	165	0.13	110	55	177	165
Serum 3.	1.03 "	25*		10.0	137	3.0	19	105	147	127
Injection.	1.07–1.33 p.m.	30	Injection of NaH_2PO_4, 20 per cent intravenously.							
Serum 4.	1.36 "	25*		9.9	152	23.1	11	100	162	134
Juice 5.	1.33–2.28 p.m.	6.4	2.54	13.4	168	2.1	102	65	181	169
Serum 5.	2.35 "	25*		12.4†	141	19.0	12	98	155	129

* Amount of whole blood.

† Potassium value possibly too high because of hemolysis.

The other constituents of both fluids were but little affected by the injection.

In Table IV are given the results of another potassium chloride injection which are similar to those of the previous experiment. The rise in the concentration of potassium in the serum after in-

jection was again delayed but the rise in the juice appeared earlier and was of the same magnitude as in the serum. Other values were unaffected by the injection.

Table IV also shows the composition of serum and pancreatic juice before and after the intravenous injection of a 20 per cent solution of sodium acid phosphate. Before the injection the concen-

TABLE V.

Injection of Calcium Chloride.

Dog 22, weight 7.2 kilos.

Sample.	Time.	Amount.	Total solids.	mM per liter.						
				Ca	Na	K	Cl	HCO_3	Na + K + Ca	Cl + HCO_3
		cc.	gm. per 100 cc.							
Juice 1.	10.33–11.04 a.m.	11.4	3.15	1.0	158	6.3	51	100	165	151
" 2.	11.04–11.50 a.m.	9.5	2.22	0.9	159	4.0	43	111	164	154
Serum 1.	11.50 "	35*		2.4	144	4.3	112	22	151	134
Injection.	12.09–12.18 p.m.	10		Injection of $CaCl_2$, 10 per cent intravenously.						
Serum 2.	12.21 "	20*		8.6	137	4.4	121	18	150	139
Juice 3.	12.19-1.10 p.m.	9.5	2.25	2.1	146	7.4	67	91	156	158
" 4.	1.10–2.53 p.m.	10.4	1.88	1.7	153	5.2	65	95	160	160
Serum 3.†	2.35 "	20*		4.7	123		118	13		131

* Amount of whole blood.

† Hemolysis.

tration of phosphate in the juice was one-twentieth that of the serum. (The high phosphate content of the normal serum of this animal was probably caused by previous ergosterol feeding experiments.) After injection, though both fluids contained a higher concentration of phosphate, the juice contained one-tenth as much as the serum. The injection of the acid solution also caused the bicarbonate of the serum to be halved with no appreciable

TABLE VI.

Injection of Calcium Chloride, Magnesium Chloride, and Sodium Acid Phosphate.

Dog 18, weight 24 kilos.

Sample.	Time.	Amount.	N	mM per liter.					
				Ca	Mg	PO_4	HCO_3	Cl	HCO_3 + Cl
		cc.	gm. per 100 cc.						
Juice 1.	10.59–11.29 a.m.	7.2	0.14	1.0	0.3	0.5	99	53	152
Serum 1.	11.30 "	15*		2.6	1.1	1.8		110	
Juice 2.	11.30 "–12.15 p.m.	9.0	0 23	1.0	0.3	0.3	98	53	151
Injection.	12.24–12.44 p.m.	24		Injection of $CaCl_2$, 10 per cent intravenously.					
Serum 2.	12.50 "	15*		6.3	0.9	2.0		124	
Juice 3.	12.53–1.04 p.m.	2.2					75	75	150
" 4.	1.04–1.21 p.m.	6.1		1.7	0.3	0.3	99	57	156
" 5.	1.21–1.44 p.m.	6.5	0.18	1.4	0.2		101	53	154
Serum 3.	1.45 "	15*		5.1	1.0	2.7		123	
Juice 6.	3.15–4.18 p m.	6.3	0.23	2.3	0.4		85	69	154
Serum 4.	4.00 "	15*		4.6	1.3				
Injection.	4.22–4.40 p.m.	24		Injection of $MgCl_2$, 10 per cent intravenously.					
Serum 5.	4.47 "	15*		4.7	8.0				
Juice 7.	4.50–5.09 p.m.	6.4	0.17	1.2	1.2		106	55	161
" 8.	5.09–5.36 p.m.	8.1	0.20	1.3	1.1	0.4	101	59	160
Serum 6.	5.35 "	15*		4.0	5.2			125	
Juice 9.	8.15–8.45 p.m.	5.6		1.7	1.0	0.6	92	69	161
Serum 7.	8.25 "	15*		4.0	4.0	4.0		129	
Injection.	8.45–9.37 p.m.	80		Injection of NaH_2PO_4, 20 per cent intravenously.					
Serum 8.	9.43 "	15*		3.0	3.7	21.2		117	
Juice 10.	9.49–10.00 p.m.	4.8		0.9	0.7	2.6	90		
" 11.	10.00–10.35 p.m.	9.1	0.26	0.9	0.7	2.6	88	70	158

* Amount of whole blood.

change in the juice bicarbonate, which is in agreement with the results reported in the previous paper.

In Table V are presented the results of an intravenous injection of a 10 per cent calcium chloride solution. Before the injection the calcium content of the juice was about one-half that of the serum. After the injection the calcium concentration of the juice was about one-fourth that of the serum, though the total concentration of calcium in both fluids was markedly increased. There was an unexplainable decrease in the sodium content of the serum after the injection which was also manifested in the juice. Other constituents showed the same relations found in the previous experiments.

In Table VI are given the results of the injection of three different salts: calcium chloride, magnesium chloride, and sodium acid phosphate, into the same animal. The results of the injections of calcium chloride and sodium acid phosphate are similar to those of previous experiments. Before the injection of the 10 per cent magnesium chloride solution the concentration of magnesium in the juice was about one-third that of the serum. After the injection the content of magnesium in both fluids increased, but the concentration in the juice was about one-sixth that in the serum.

It is interesting to note in this experiment the increase in inorganic phosphate of the serum caused by the injections of calcium and magnesium chloride. Since calcium and magnesium form insoluble salts with phosphate, the opposite result might be expected. This same observation has also been made by Salvesen, Hastings, and McIntosh (7).

DISCUSSION.

The inorganic composition of pancreatic juice reported here is similar to that to be reported in a paper by Johnston and Ball (8) for juice obtained from dogs with permanent fistulas. This would seem to indicate that the pancreas was functioning normally in the experiments reported in the present paper, despite the possible unfavorable action of anesthetic, operation, and experimental procedures.

The concentrations of sodium and potassium in pancreatic juice are similar to their concentrations in the serum of the same

animal. Experimental variations in the concentrations of these ions in the serum are reflected quantitatively in the pancreatic juice. Thus it would appear that the pancreatic tissue is freely permeable to the sodium and potassium of the blood serum. On the other hand, calcium, magnesium, and phosphate ions appear to filter through the pancreatic tissue only with difficulty. The concentrations of these ions in pancreatic juice are much lower than in the serum, and large increases in their concentrations in the serum are attended by only slight increases in the juice.

The quantitative composition of pancreatic juice in respect to the cations is similar to that of cerebrospinal fluid and bile. If the blood serum is the immediate source of the cations of all these fluids it would seem that the membranes controlling the composition of these fluids are alike in their free permeability to sodium and potassium and their poor permeability to calcium and magnesium ions.

The low calcium concentration of cerebrospinal fluid as compared to serum has been explained by some workers as due to the fact that only the diffusible calcium of the serum appears in the fluid. If the low calcium content of pancreatic juice is explained in the same manner, then the intravenous injection of calcium chloride, presumably causing a large increase in the diffusible calcium of the serum, should cause a correspondingly large increase in the calcium of the juice. That this does not occur would indicate that low concentrations of calcium in the juice are the result of the slight permeability of pancreatic tissue to this ion.

The total organic matter in any particular juice produced by secretin appears to depend on the previous activity of the gland. If a resting pancreas is stimulated, the juice evoked will contain more organic material than that obtained from a gland that has just previously been actively secreting. This would seem to indicate that the enzymes of the juice are produced independently of the fluid. The decreasing organic content of juice during a prolonged secretion provoked by secretin, might then be attributed to a flushing out of the gland's preformed enzymes at a rate far above the rate of formation. This would agree with Mellanby's (9) view that secretin controls the production of only the alkaline fluid, the enzyme production being controlled possibly by a nervous mechanism.

XUM

The organic matter usually has a nitrogen content of 16 per cent, though as stated above, percentages ranging from 11 to 28 have been obtained. These variations are well beyond the experimental error of the micro method. Values as high as 28 per cent would suggest the possibility of the presence of some material such as urea. It is hoped that this peculiarity can be further investigated in the future.

TABLE VII.

Total Inorganic Composition of Juice and Serum of Control Periods.

Dog No.	Sample.	Concentration in mM.			
		Cations:		Anions:	
		Per volume.	Per kilo water.	Per volume.	Per kilo water.
19	Juice.	162	165	158	161
	Serum.	147	160	137	149
20	Juice.	163	166	160	163
	Serum.	145	158	139	151
22	Juice.	165	168	151	154
	Serum.	152	165	136	148
23	Juice.	159	162	157	160
	Serum.	151	164	135	147
24	Juice.	168	171	159	162
	Serum.	155	168	132	144
27	Juice.	155	158	142	145
	Serum.	144	157	130	141

Although freezing point determinations indicate that pancreatic juice and serum have identical osmotic pressures, the total inorganic cation and anion concentration per volume of juice is greater than that of the serum. However, if concentrations are expressed in the accepted manner used by those studying osmotic pressure relations, namely per kilo of water, this apparent discrepancy diminishes. In Table VII are shown the total inorganic cation and anion concentrations of juice and serum calculated per volume and per kilo of water. The water content of juice in these calculations

has been assumed to be 98 per cent (Johnston and Ball (8)) and that of the serum 92 per cent (Kerr (2)). The total cation concentration is the sum of sodium, potassium, calcium, and magnesium. In some cases where calcium and magnesium were not actually determined, average normal values for juice and serum were assumed. Total anions represent the sum of the concentrations of chloride, bicarbonate, and phosphate ions. Here also, in some cases, an average normal value for phosphates in both fluids was assumed. These assumptions can cause but a small error in the total, because of the relatively small figures involved.

It will be seen that, when concentrations for both fluids are expressed per kilo of water, the concentrations of base in the two fluids are in good agreement. The concentrations of acid radicals show wider variations, undoubtedly due, in part at least, to the variation in base bound by protein which is present in unequal concentrations in serum and juice.

It will also be noticed in Table VII that in the juice the excess of inorganic base over inorganic acid radicals is quite variable. The variations run from an excess of 3 to 14 millimols per liter. Some of this variation is most certainly due to experimental error. However, the average excess of base in these six experiments is about 8 millimols per liter. This excess of base cannot be bound by the small amount of protein in the juice if the combining capacity of the juice proteins is similar to that of serum proteins. Either the binding power of juice protein is greater than that of the serum protein or other organic radicals are also present to bind part of this base.

Since the concentration of protein in the serum is greater than in the pancreatic juice, there is a possibility that a Donnan effect would exist. No conclusions can be drawn for the individual ions, for in some cases, chloride and bicarbonate, the concentrations in the two fluids are widely different. However, if the concentrations of total anions per kilo of water in both fluids are considered, then a relation exists that would suggest a possible Donnan equilibrium. The pancreatic juice contains an excess of anions over the serum, of a magnitude similar to that which can be calculated on the basis of the different protein content of the two fluids. If, however the concentration of total cations is now considered in both fluids, it will be seen that there is a relation just the opposite

of that which would be expected if a Donnan equilibrium existed; the total cations are present in greater instead of lesser concentration in the juice than in the serum. Hence no definite conclusions can be drawn with regard to a Donnan effect.

SUMMARY.

1. Pancreatic juice and blood serum have identical osmotic pressures.

2. Concentrations of sodium and potassium expressed per kilo of water are similar in the two fluids. The injection of these ions into the blood stream increases their concentrations in both fluids to the same degree. It is therefore concluded that the pancreatic tissue is freely permeable to sodium and potassium.

3. Calcium, magnesium, and phosphate were found in pancreatic juice in concentrations one-twentieth to one-fourth those in blood serum. The intravenous injection of salts yielding these ions in amounts sufficient to increase greatly their concentrations in the serum caused but little increase in their concentrations in the juice. The conclusion is drawn that the pancreas is but slightly permeable to calcium, magnesium, and phosphate ions.

4. The concentration of total organic matter in the juice appears to depend upon the state of activity of the gland previous to the secretion of the juice.

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A NOTE ON THE DETERMINATION OF IRON IN MILK AND OTHER BIOLOGICAL MATERIALS.

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In a previous paper, Elvehjem and Hart (1), attention was called to the interference of phosphate in the determination of iron in materials of high phosphate content, and a method was outlined to eliminate the difficulty. This method was developed particularly for the determination of iron in milk. Since that time several papers have been published giving methods for the determination of iron in biological materials. In all of the proposed methods, which are modifications of the sulfocyanate method, the purpose has been to make the procedure more adaptable to the estimation of iron in blood and animal tissues.

The suggested improvements have consisted either of a more rapid method of oxidizing the organic matter, or a method of developing the color more completely by the use of organic solvents. Smirk (2) suggested the use of ammonium persulfate and nitric acid for the destruction of organic matter. Fowweather (3) used perhydrol in sulfuric acid solution, while Kennedy (4) replaced the perhydrol with 60 per cent perchloric acid. Any of these reagents may be used successfully for blood and animal tissues which are high in iron and require only a small sample for an analysis. The large amount of milk required for a single iron estimation prevents the use of these methods for the oxidation, especially those which employ sulfuric acid, because of the formation of a precipitate of calcium sulfate. Therefore ashing seems to be the most feasible method for preparing milk for analysis.

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The more recent workers have used acetone for the extraction of the colored ferric sulfocyanate from the interference of the materials present in the aqueous solution. Kennedy (4) suggested the use of amyl alcohol in place of acetone, because it is less volatile and insures a more constant volume after extraction. He also presented figures to show that the addition of phosphate did not cause a fading of the color when amyl alcohol was used, but rather increased the color to the extent of the iron impurity in the salt added.

Unfortunately the proposed improvements are not directly applicable to the determination of iron in milk, and therefore cannot be used to shorten the method proposed in an earlier paper. When Kennedy's tests were repeated with phosphate, it was found possible to recover the iron quantitatively in the presence of orthophosphate salts when amyl alcohol was used. However, when the same procedure was applied to the ash of milk the usual characteristic color fading was observed. Similar results were obtained when a mixture of iron and phosphate was ignited before it was tested for iron. Undoubtedly changes took place in the phosphate molecule during the ashing process which did not occur with nitric and perchloric acids used by Kennedy for the oxidation of the tissues. Since salts of orthophosphoric acid are converted to pyrophosphates at temperatures above 200°, some of the phosphate is very probably present in the ash as the higher oxide.

The inhibitory effect of pyrophosphate on iron was realized during the study of the influence of pyrophosphates on iron when used as a catalyst in the oxidation of cysteine. Warburg (5) has shown that the catalytic effect of iron in the oxidation of cysteine is completely retarded in the presence of a small amount of pyrophosphate. He has further shown that no blue color is formed when potassium ferrocyanide is added to a small amount of iron in the presence of pyrophosphate, but that the characteristic reddish brown color is obtained when the same reagent is added to copper in the presence of pyrophosphate. Pyrophosphate, therefore, prevents the reaction between ferrocyanide and iron, but does not influence the reaction between the cyanide and copper. Pyrophosphate also inhibits the reaction between iron and potassium sulfocyanate. It not only inhibits the reaction, but causes

a decomposition of the colored ferric sulfocyanate after it has been formed. The extraction of the color by amyl alcohol does not prevent this action of the pyrophosphate.

A simple method of converting the pyrophosphate present in the ash of milk, and any other materials high in phosphate, would permit the estimation of the iron directly in the solution of the ash without removal of the phosphorus. Eggleton and Eggleton (6) used N HCl at boiling temperature for the hydrolysis of pyrophosphate in muscle extract, but this procedure was found inadequate for the large amounts present in milk ash. After several methods had been tried, that of boiling with dilute NaOH was found to be most efficient.

If 0.1 mg. of Fe and 100 mg. of sodium pyrophosphate are dissolved in dilute HCl and enough NaOH added to make the solution distinctly alkaline, the iron remains in solution. Iron is soluble in an alkaline solution in the presence of excess pyrophosphate. If the solution is boiled for some time, a precipitate of ferric orthophosphate begins to appear, showing that the pyrophosphate has been hydrolyzed. This also affords an explanation of the prolonged boiling with dilute NaOH necessary to precipitate the iron in our earlier method. Enough pyrophosphate remained in the filtrate from the ammonium molybdate precipitation to hold the iron in solution, and the iron could be precipitated only after hydrolysis of the pyrophosphate.

After the solution has been boiled slowly for about 1 hour, the iron can be determined quantitatively by making the solution acid, adding potassium sulfocyanate, and extracting the color with amyl alcohol. In the actual determination the solution was made to a volume of 50 cc. after the acid had been added and the iron determined in 10 cc. aliquots. The results of the estimations in two such series are shown in Table I.

These figures show that no difficulty is encountered after the pyrophosphate is hydrolyzed providing the colored ferric sulfocyanate is extracted with amyl alcohol. There is enough orthophosphate present in the hydrolyzed solution to cause color fading unless the alcohol is used.

In Table II figures are given to show that the same results are obtained in the analysis of milk powder. In every case there is a complete recovery of added iron whether the addition was made

before or after ashing. The milk powder contained 0.00226 per cent Fe. In addition to milk and milk products, this procedure may be used advantageously for other materials high in phosphate.

The complete determination of iron in milk or any biological material which forms pyrophosphate upon ashing may be outlined as follows: Samples containing 0.1 to 0.3 mg. of Fe are evaporated to dryness (in the case of milk) and thoroughly ignited.

TABLE I.

Recovery of Iron in Pyrophosphate Solutions after Hydrolysis.

Sample No.	Fe per 10 cc.	Fe addition per 10 cc.	Total Fe recovered.
	mg.	*mg.*	*mg.*
1	0.02	None.	0.0192
2	0.02	"	0.0197
3	0.02	"	0.0202
4	0.02	0.02	0.0398
5	0.02	0.01	0.0302
6	0.01	0.02	0.0303

TABLE II.

Recovery of Added Iron in the Analysis of Milk Powder.

Sample.	Volume of aliquot.	Fe addition per aliquot.	Total Fe recovered.
	cc.	*mg.*	*mg.*
3 gm. milk powder alone made to 50 cc. volume after hydrolysis.	10	None.	0.0136
	20	"	0.0260
	5	0.02	0.0266
	10	0.02	0.0332
3 gm. milk powder + 0.1 mg. Fe made to 50 cc. volume after hydrolysis.	10	None.	0.0334
	5	"	0.0168
	5	0.02	0.0365

The ash is taken up in dilute HCl and any insoluble residue filtered off. Enough 40 per cent NaOH (iron-free) is added to make the solution distinctly alkaline, and the solution is boiled 1 hour. In the case of milk a precipitate of calcium phosphate will naturally be formed, but this does not interfere with the reaction. When the hydrolysis is completed the solution is made acid with HCl and diluted to a volume of 50 cc. The colorimetry is then carried out as outlined by Kennedy. 1 cc. of standard iron solution

equivalent to 0.1 mg. of Fe is dissolved in 5 cc. of HCl and diluted to 50 cc. Aliquot portions of 10 cc. each of the unknown and the standard solutions are placed in a stoppered cylinder. 5 or 10 cc. of amyl alcohol and 5 cc. of 20 per cent potassium sulfocyanate are added to each cylinder and the mixture thoroughly shaken. The colored layers of amyl alcohol are then compared in a colorimeter.

SUMMARY.

1. A revised method for the quantitative determination of iron in milk outlined.

2. This method eliminates the difficulties encountered due to the formation of pyrophosphate during the ignition of milk samples.

3. The method may also be used for the determination of iron in other biological materials rich in phosphorus.

I wish to acknowledge the advice and encouragement of Sir Frederick Gowland Hopkins in whose laboratory this work was completed.

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ON THE CHEMICAL COMPOSITION OF THE ISLANDS OF LANGERHANS IN THE MONKFISH (LOPHIUS PISCATORIUS, L.).

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INTRODUCTION.

There is in general little known about the relationship between the chemical composition of an organ and its function. This is particularly the case for the digestive glands which produce enzymes. In regard to the pancreatic gland there is, however, one peculiarity in its composition, which is very striking; namely, its high content of nucleic acids. The pancreas contains relatively from 2 to 3 times more pentose nucleic acids than do the other digestive glands. This tends to indicate that the nucleic acids play a significant rôle in the gland. Since the pancreatic juice is strongly alkaline, equivalent approximately to a 0.1 N bicarbonate solution, and since the organic phosphates have a very high buffering capacity, some relationship might be assumed.

Hammarsten and the author[1] suggested the hypothesis that the nucleic acids in pancreas can act *in loco* as indiffusible, strongly buffering substances, capable of preventing rapid changes in the reaction of the gland during secretion. In the salts of nucleic acids a considerable amount of alkali is stored, which can be mobilized during the secretory phase. The free phosphates and the carbonates cannot accumulate in the cells to the same extent as the organic phosphates, because the former are easily diffusible; they consequently cannot exert there the same buffering effect. If this is indeed one of the rôles of the nucleic acids in the pan-

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[1] Hammarsten, E., and Jorpes, E., *Acta med. Scand.*, **68**, 205 (1928).

creas, it seems reasonable to assume that these acids should accumulate particularly in the zymogenous part of the gland, namely in the acini.

As the function of the islands of Langerhans is quite different from that of the acini, these islands may also have a different chemical composition, although they develop during the embryonic life from the same tubuli of the intestinal epithelium as do the acini.[2] The detection of a difference in the chemical composition of the two parts of the pancreas would therefore be of interest. It might also be of value in the discussion concerning the transformation of zymogenous tissue into islet tissue and *vice versa* in the postembryonic life. Inasmuch as the parenchymatous organs have a very definite content of phospholipoids,[3] nucleic acids,[4] etc., a marked difference in the chemical composition of the acini and of the islands of Langerhans would exclude any transformation of one part into the other in the postembryonic life at least in that species. Such a transformation was claimed to be possible in transition from one digestive phase to another (Vincent and Thompson[5]). This view is scarcely supported by the histologists.

Previous investigations in this field so far have dealt only with the pentose content of the two parts of the gland. In 1923 Berkeley,[6] using Grund's modification of Tollens' reaction method, compared the yield of furfuraldehyde from the lipoid-free dry substance of the islands of Langerhans with that from the acinous pancreatic tissue of the ling cod (*Ophiodon elongatus*, Girard). This comparison was possible because the islands of Langerhans form in most of the Teleostei fishes a separate organ, in some of them well encapsulated, completely free of acini, and easily accessible.[7] Berkeley found a slightly higher pentose content in the islet tissue than in the mesenterial tissue, which was sup-

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[3] Javillier, M., and Allaire, H., *Bull. Soc. chim. biol.*, **7**, 486 (1923); **8**, 924 (1924); **9**, 772 (1927).

[4] Jorpes, E., *Acta med. Scand.*, **68**, 253, 503 (1928).

[5] Vincent, S., and Thompson, F. D., *Internat. Monatschr. Anat. u. Physiol.*, **24**, 61 (1907).

[6] Berkeley, C., *J. Biol. Chem.*, **58**, 611 (1923–24).

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posed to be the zymogenous part. He did not call much attention to this slight difference, but he emphasized the possibility that the high pentose content of the pancreas might be due to its insular part. In a different way Hammarsten, Hammarsten, and Olivecrona[8] later obtained quite different results. After ligation of the two or three main pancreatic ducts in dogs, which caused complete disappearance of the zymogenous tissue without any diabetic symptom in the animals, they determined, using the same method as Berkeley, the pentose content in the pancreatic residue. They did not find more than a trace of furfuraldehyde in the connective tissue, which contained a large amount of islands.

This being the case, a further investigation seemed desirable. The present study was undertaken with the view of comparing the islet tissue of some bony fishes with the normal pancreatic tissue in the selachian fishes. As experimental material the islet tissue of the monkfish (*Lophius piscatorius*, L.) was chosen. The corresponding glands from the summer flounder (*Paralichthys dentatus*, Girard) were also analyzed.

In *Lophius* rather large glands are found, a large one adjacent to the ductus cysticus not far from the gallbladder and a smaller one in the neighborhood of the pylorus. These two glands can easily be decapsulated. Their parenchymatous part is pure islet tissue, whereas the capsule contains more or less of acini. The acini form, however, only the smaller part of the connective tissue, in which they are imbedded. They are not tightly agglomerated to form glands like the pancreas of the selachian fishes and of the mammals. This therefore makes the comparison of the composition of the islands of Langerhans with that of the acini in one animal impossible. For comparison the normal pancreatic gland in several selachian fishes had to be analyzed.

DISCUSSION.

In order to interpret the findings the composition of the Langerhans organ in *Lophius* has to be compared both with that of the normal pancreas of selachian fishes and with the capsular tissue surrounding the gland as well as with the liver of the same fish.

[8] Hammarsten, E., Hammarsten, G., and Olivecrona, H., *Acta med. Scand.*, **68**, 215 (1928).

TABLE I.

Organ analyzed.	Kind of fish.	No.	Dry substance.	Total P.		Pentoses (furfuraldehyde × 1.56).		Total P on lipoid-free dry substance.	Pentoses on lipoid-free dry substance.	Chloroform-soluble material in the alcohol-ether extract (on basis of fresh organ).	Lipoid P taken up with chloroform from the dry alcohol-ether extract (on basis of fresh organ).
				On fresh organ.	On dry substance.	On fresh organ.	On dry substance.				
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Pancreas.	Dogfish (*Squalus acanthias*, L.).	1		0.357		0.331					
		2	23.06	0.377	1.64						
		3	23.65			0.305	1.29				
		4		0.431		0.305					
		5		0.424		0.284					
		6	23.12	0.375	1.62	0.323	1.40				
		7		0.433							
		8						1.56	2.51		
	Common skate (*Raia erinacea*, Mitchill).	1		0.464				1.64	1.66(?)	3.54	0.093
		2						1.75	2.65	4.91	
		3	24.00					1.49	2.61	3.63	0.080
	A big skate (*Raia diaphanes*, Mitchill).	1	23.70	0.356	1.50			1.27	1.80	4.18	0.086
	A shark.	1	23.16			0.282	1.22			4.81	
				0.402		0.305		1.54	2.25	4.21	0.086

Islet tissue of Langerhans.	Monkfish (*Lophius piscatorius*, L.).	1		0.312						4.42	0.088
		2		0.326							
		3		0.302							
		4	14.80	0.262	1.77	0.081	0.54				
		5		0.283				1.02	0.73	5.28	0.117
		6	16.90	0.304	1.79						
		7		0.337							
		8		0.335							
		9		0.317							
		10		0.303		0.104		1.18	0.87		
		11		0.317		0.126		0.93		4.90	0.102
		12						0.99	0.93	4.68	0.098
		13							0.93		
				0.309		0.104		1.03	0.87	4.82	0.101
Capsular tissue surrounding the island gland.	Monkfish.	1	13.70	0.253	1.85	0.136	0.99	0.92	1.38	2.72	0.057
		2	14.10			0.153	1.08	0.97	1.60	2.02	0.047
		3	15.40	0.209	1.39	0.154	1.00	1.03	1.16		
		4						1.11		3.67	
		5						0.92	1.20		
		6						1.13	1.14		
						0.146		1.01	1.30	2.81	0.052
Liver.	Monkfish.	1						1.01	0.64		
		2						0.885	0.74		
	Dogfish.	1				0.096					

It becomes evident from Table I that the islet tissue in *Lophius* has a chemical composition which differs distinctly from that of the normal pancreatic tissue in selachian fishes in regard to the phosphorus and pentose content of the lipoid-free residue and to the content of lipoid phosphorus of the fresh organ. The phosphorus content of the lipoid-free dry substance is only two-thirds of that found in the pancreas of selachian fishes. It is of the same order of magnitude as the phosphorus content of the lipoid-free liver substance of the *Lophius* itself. The comparison with the liver is very valuable when no pancreas is to be found, as in the bony fishes. In mammals the percentage of pentoses in the liver is much lower than in the pancreas. Analyses of the liver of the dogfish, of which only one is given in Table I, showed this to be true here also.

The low pentose content, 0.87 per cent found in the lipoid-free islet tissue, differs strikingly from the value 2.25 per cent found in the lipoid-free dry substance of pancreas from other fishes and approaches the figures found in the liver, *viz.* 0.64 and 0.74 per cent.

It is possible, however, that the islands of Langerhans contain some labile compound, which could to a certain extent destroy the furfuraldehyde formed. If this is not the case, it results from Table I that the capsular tissue, surrounding the island organs, contains more pentoses than do the islands, the yield of furfuraldehyde being greater both with fresh tissue and with lipoid-free residue. The difference in yield of furfuraldehyde is most probably due to a real accumulation of pentose nucleic acids in the capsular tissue, which contains more or less of acini. The bulk of the capsule is, however, mainly connective tissue.

More striking is another difference; namely, that in the content of lipoids and lipoid phosphorus in the islands and in the capsular tissue. The latter contains only half the amount found in the fresh islet tissue. This difference also appears in comparing the islet tissue with the normal pancreas. Although the islet tissue in *Lophius* contains only around 16 per cent of dry substance, the content of lipoid phosphorus of the fresh organ is higher, namely 0.101 per cent (as found in four samples), than in the selachian pancreas, 0.086 per cent (as found in three samples), which contains 23 per cent of dry substance. The high

lipoid content of the islands of Langerhans is most probably due to a rich supply of nerves. The finding is therefore in accordance with the common view that the function of the islands is at least partly regulated by the nervous system.[9]

The analyses of the decapsulated islet tissue of the summer flounder showed a somewhat higher content of pentoses, namely 0.214 per cent in the fresh organ (or 0.92 per cent calculated for dry substance), which figures also are lower than those found for the normal pancreas of selachian fishes.

EXPERIMENTAL.

In order to estimate the nucleic acid content of the glands, the fresh glands and their lipoid-free dry substance were analyzed for phosphorus and pentoses. The same technique and the same methods were used for the analytical work as in an earlier work on the nucleic acid content of parenchymatous organs.[4]

All phosphorus determinations were made according to the Pregl-Lieb method. The pentose determinations were made with Hoffman's micro method. The lipoid-free material was prepared by Soxhlet extraction with 95 per cent alcohol during 24 to 36 hours, followed by extraction with ether during several hours. For determination of the lipoid phosphorus, the extract was carefully dried under reduced pressure on a boiling water bath. The residue was extracted several times with chloroform and the chloroform solution filtered. The chloroform was evaporated, and incineration made with sulfuric acid.

Because of the limited amount of material available, the analytical data are rather incomplete, but give, however, satisfactory information for the aim of this discussion. They are summarized in Table I, while the analyses of the fresh decapsulated Langerhans organ of the summer flounder are as follows:

Dry substance (four samples): 23.97, 23.06, 23.53 and 22.56 (average 23.31) per cent.

Total phosphorus (five samples): 0.372, 0.370, 0.343, 0.342, and 0.352 (average 0.356) per cent.

Pentoses (two samples): 0.222 and 0.206 (mean 0.214) per cent. Calculated on dry substance mean 0.92 per cent.

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[9] Britton, S. W., *Am. J. Physiol.*, **74**, 291 (1925).

SUMMARY.

The islet tissue of Langerhans of the monkfish (*Lophius piscatorius*, L.) and the summer flounder (*Paralichthys dentatus*, Girard) has been analyzed for its content of nucleic acids and phospholipoids and compared in this respect with the normal pancreas of selachian fishes and with the mesenterial tissue surrounding the isolated Langerhans organ which is known to contain acini.

In *Lophius*, where the islands form large separate organs, these do not have the typical composition of the pancreatic tissue. The content of nucleic acid is much lower than in the normal pancreas of selachian fishes and of mammals. On the other hand the content of phospholipoids is higher.

The possibility is discussed that the high content of nucleic acids in the normal pancreas might be due to an accumulation in the acini, where the organic phosphates possibly serve as buffers during the secretion of the alkaline pancreatic juice.

I wish to express my sincerest thanks to Dr. M. H. Jacobs, Director of the Marine Biological Laboratory, for his courtesy in extending to me the facilities of the Marine Biological Laboratory and the United States Bureau of Fisheries for kindly supplying a part of the material needed for this investigation.

PURINE METABOLISM.

III. DIET AND CAGING AS FACTORS IN THE ALLANTOIN EXCRETION OF THE RABBIT.

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INTRODUCTION.

It has generally been assumed that allantoin is the terminal product of purine metabolism in most mammals with the exception of man and the anthropoid apes. Since uric acid is the end-product of purine metabolism in these species, the tissues of these animals apparently do not contain the enzyme uricase, which converts uric acid to allantoin.

Whether uric acid in man and allantoin in other mammals have the same metabolic significance cannot be decided, until more data relative to the factors which govern the excretion of allantoin have been collected. The present study is concerned primarily with the effect of diet and caging on the allantoin excretion of the rabbit. A review of the literature relative to the allantoin excretion of the rabbit indicates that the values range from 50 to 200 mg. per day (1–4). This variation cannot be attributed to analytical methods, since in most of the work, the method of Wiechowski (1) or the Handovsky (5) modification of this method has been used. In the present paper more data have been collected relative to the purine metabolism of the rabbit.

EXPERIMENTAL.

The rabbits used in these experiments were healthy males, varying in weight from 2.5 to 3.5 kilos. In most cases they were taken directly from the stock pen at the animal house and placed in a small metabolism cage in the laboratory. The stock rabbits at the animal house received a ration consisting of cracked corn,

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oats, alfalfa hay, and a small daily portion of a fresh vegetable, usually carrot. The subsequent diets of the rabbits are recorded in Tables I and II. Inasmuch as some of the experiments on an individual rabbit extend over a period of several months, it is well to state that the animals were in good physical condition at the end of the experiment unless statements to the contrary are given in the text. The appearance, appetite, and activity of the rabbits were used as criteria of their physical condition. Unless otherwise stated, the rabbits consumed all the food which was offered to them.

The urines were collected for periods of 24 hours. The urine excreted in the cage was received in a bottle containing toluene and a small amount (0.5 cc.) of glacial acetic acid. The urine remaining in the bladder at the end of the collection period was removed by gentle pressure in the region of the bladder and added to that collected in the cage. Analyses were made for total nitrogen, creatinine, uric acid, and allantoin. Creatinine was estimated by the micro method of Folin (6), total nitrogen by the Kjeldahl method, uric acid by the method of Morris and Macleod (7), and allantoin by the method of the author (8).[1] The effect of diet and caging upon the allantoin excretion of ten rabbits was studied. The duration of these experiments varied from 1 to 7 months. Since many of the data are similar two typical experiments only are presented in tabular form (Tables I and II).

DISCUSSION.

A survey of the data obtained from these experiments reveals the following points. (1) The allantoin excretion of the rabbits for the period immediately following their removal to the laboratory from the animal house was much higher than for a subsequent period following confinement on a restricted diet in the metabolism cage. (2) Corresponding to the decreased excretion of allantoin there was in most cases after caging a decreased excretion of uric acid. (3) In several experiments a marked increase in allantoin excretion was noted, if the rabbit after a period of confinement in

[1] The hydrolyses of allantoin to oxalic acid are now made in Pyrex test-tubes (200 × 25 mm.) maintained at a temperature of 115–120° in a calcium chloride bath for 1 hour and 15 minutes. Neutralization of the strong alkali and subsequent dilution to a volume of 50 cc. are performed in the same tube.

TABLE I.

Influence of Diet and Caging on the Excretion of Allantoin by the Rabbit.

Rabbit 7. Gray male. Brought from animal house October 11 and immediately placed in a metabolism cage. The rabbit was given a daily diet of 70 gm. of oats and 100 gm. of cabbage and water *ad libitum*.

Date.	Weight.	Creatinine.	Total N.	Uric acid.	Allantoin.
		Period I.			
1927	*kg.*	*mg.*	*mg.*	*mg.*	*mg.*
Oct. 12	3.1	151	916	21.0	227
" 13	3.0	151	1117	20.0	234
" 14	3.0	163	1100	17.0	183
Average				19.0	215
Period II. From Oct. 14 to Nov. 1 rabbit confined in metabolism cage, on same diet.					
Oct. 30	2.9	144	1302	11.0	133
" 31	2.9	143	1260	12.0	136
Nov. 1	2.9	144	1302	12.0	151
Average				11.7	140
Period III. From Nov. 1 to Jan. 6 rabbit still confined in small metabolism cage and diet of previous periods continued.					
Jan. 4	2.5	93	706	7.6	48
" 5	2.5	88	798	7.5	52
" 6	2.5	102	873	11.0	52
Average				8.3	51
Period IV. Jan. 7 rabbit removed from metabolism cage and placed in pen approximately 4 × 10 ft. in area. Diet of previous period continued. Jan. 31 rabbit returned to metabolism cage, diet remaining the same.					
Feb. 1	2.8	114	924	10.0	98
" 2	2.8	120	832	8.0	88
" 3	2.8	104	638	7.0	81
Average				8.3	89

TABLE I—*Concluded.*

Date.	Weight.	Creatinine.	Total N.	Uric acid.	Allantoin.
Period V. From Feb. 8 to Feb. 26 rabbit kept in metabolism cage and diet limited to 300 gm. carrots per day with water *ad libitum*.					
1927	*kg.*	*mg.*	*mg.*	*mg.*	*mg.*
Feb. 23	2.7	120	647	8.7	48
" 24	2.7	114	588	9.0	43
" 25	2.7	111	697	8.7	38
" 26	2.7	116	598	9.9	40*
Average.....				9.1	42
Period VI. From Feb. 28 to Apr. 1 rabbit kept in metabolism cage and given 70 gm. oats and distilled water *ad libitum*.					
Mar. 28	2.5	105	687	9.0	100
" 29	2.5	102	733	8.3	109
" 30	2.5	98	672	8.4	92
Apr. 1	2.5	111	773	9.4	101
Average.....				8.8	100
Period VII. From Apr. 3 to Apr. 21 rabbit confined in metabolism cage and offered diet of 40 gm. oats and 100 cc. milk.					
Apr. 16	2.7	122	1075	6.4	35
" 17	2.7	122	983	6.4	47
" 18	2.7	122	907	6.3	51
" 19	2.7	116	949	5.8	50†
" 20	2.6	122	882	8.1	68‡
" 21	2.6	125	814	6.1	67§

* The daily excretions of uric acid and allantoin respectively for the following 3 days were 8.0, 8.1, 8.7 mg., and 83, 88, and 90 mg.

† The milk contained 500 mg. of added calcium chloride.

‡ 70 gm. of oats and 50 cc. of milk, containing 500 mg. of added calcium chloride were consumed.

§ 70 gm. of oats and 25 cc. of milk containing 750 mg. of added calcium chloride were consumed.

the metabolism cage was given the freedom of a larger pen. (4) Marked changes in the allantoin excretions of some rabbits were observed, following a change in the diet, although other factors remained the same.

TABLE II.

Influence of Diet and Caging on the Excretion of Allantoin by the Rabbit.

Rabbit 9. Light brown male. Brought from animal house Feb. 9 and immediately placed in a metabolism cage. The rabbit was given a daily diet of 50 gm. of oats and 100 gm. of carrots with water *ad libitum*.

Date.	Weight.	Creatinine.	Total N.	Uric acid.	Allantoin.
		Period I.			
1928	*kg.*	*mg.*	*mg.*	*mg.*	*mg.*
Feb. 10	2.1	100	739	10.7	140
" 11	2.1	92	798	10.4	129
" 12	2.1	102	739	16.0	147
" 13	2.1	115	823	12.0	149
Average				12.3	141
Period II. From Feb. 15 to Apr. 6 rabbit confined in metabolism cage on same diet.					
Apr. 3	2.2	100	739	12.0	50
" 4	2.2	98	697	12.0	44
" 5	2.2	100	663	11.0	43
" 6	2.2	99	613	10.5	42
Average				11.4	45
Period III. Apr. 7 the 100 gm. of carrots in daily ration were replaced by 100 gm. of cabbage. From Apr. 7 to May 4 rabbit remained in metabolism cage on diet of 100 gm. cabbage and 50 gm. oats.					
May 1	2.0	97	806	9.9	96
" 2	2.0	101	773	10.0	99
" 3	2.0	102	773	8.9	90
" 4	2.0	98	789	8.7	90
Average				9.4	94
Period IV. May 5 rabbit taken from metabolism cage and confined in larger pen, on same diet. May 22 rabbit returned to metabolism cage, diet remaining unchanged.					
May 22	1.9	96	857	10.8	106
" 23	1.9	97	941	11.0	117
" 24	1.9	97	667	11.0	128
Average				10.9	117

TABLE II—*Concluded.*

Date.	Weight.	Creatinine.	Total N.	Uric acid.	Allantoin.
Period V. From May 25 to June 30 rabbit still confined in metabolism cage on same diet.					
1928	*kg.*	*mg.*	*mg.*	*mg.*	*mg.*
June 27	1.9	96	781	4.4	33
" 28	1.9	99	806	4.1	32
" 29	1.9	91	840	4.5	52
" 30	1.9	97	974	5.4	55
Average				4.6	44
Period VI. From July 1 to July 12 rabbit confined in metabolism cage but previous diet was supplemented with 100 cc. milk. This was always eagerly consumed in addition to cabbage and oats.					
July 9	1.95	106	882	5.5	43
" 10	1.95	105	697	4.3	60
" 11	1.95	104	655	4.7	64
" 12	1.95	108	890	4.7	73
Average				4.8	60

The marked decrease in the excretion of allantoin by the rabbit after confinement in a small metabolism cage is well illustrated in the experiments with Rabbit 7 (Table I, Periods I, II, and III). The allantoin excretion of this rabbit progressively decreased from 215 to 51 mg. per day during a 3 months confinement in a metabolism cage on a diet of oats and cabbage. The average excretion of uric acid during this same period decreased from 19 to 8.3 mg. per day. It is to be noted that there was also a significant drop in the excretion of creatinine during the period from November 1st to January 4th, coincident with the loss in weight of the rabbit. An examination of Table I as a whole, shows that there is little correlation between the excretion of total nitrogen and allantoin.

Experiments similar to those reported in Table I (Periods I, II, and III) have been made on each of the ten rabbits that have been studied. In every case, there was a progressive decrease in the daily excretion of allantoin, when the rabbit was confined in a small metabolism cage on a restricted diet. In some experi-

ments, it was observed that after a period of caging, there was a decrease in the excretion of allantoin of the rabbit, without a corresponding decrease in uric acid excretion. The results obtained with Rabbit 9 (Table II, Periods I and II) were of this type. In this particular experiment, the rabbit received a diet of oats and carrots instead of oats and cabbage, as noted in the experiments recorded in Table I. Since similar results were obtained with rabbits receiving a diet of oats and cabbage, it was concluded that individual differences in the purine metabolism of the rabbits, rather than a dietary factor, must be an explanation of these results.

It was thought probable that if the rabbits received a more varied diet immediately after caging, the decrease in allantoin excretion might not be obtained. Accordingly, two rabbits (Rabbits 14 and 15) were caged and given a diet consisting of 40 gm. of oats, 35 gm. of cracked corn, 50 gm. of carrots, and 50 gm. of cabbage. After 1 month of confinement in the metabolism cage on this diet, the excretions of allantoin were less than half of that observed for the initial period of caging.

To ascertain whether the decreased allantoin excretion just discussed might be due to the close confinement in the metabolism cage, Rabbit 7 (Table I, Period IV) was removed from the metabolism cage to a pen approximately 8 by 10 ft. in area. The rabbit was kept in this pen for 24 days on the diet of the previous period. The average daily excretion of allantoin for the first 3 days after reconfinement in the metabolism cage was 89 mg. as compared to 51 mg. of the previous period. A similar result was obtained in an experiment with Rabbit 9 (Table II, Periods III, IV, and V). It is to be noted in the experiment on the latter rabbit, that the excretions of allantoin and uric acid were decreased considerably after reconfinement in the metabolism cage on the diet of oats and cabbage (Table II, Period V). Apparently in the case of these two rabbits, the greater activity which was made possible by the larger pen has led to a marked increase in the excretion of allantoin. In a similar experiment on a third rabbit it was found that during the period of greater activity, the allantoin excretion was increased approximately 10 per cent, and the uric acid excretion doubled.

That the excretion of allantoin by the rabbit is markedly influ-

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enced by diet has been demonstrated in experiments on each of the ten rabbits that were used in this work. As typical illustrations, the experiments with Rabbit 7 (Table I, Periods V and VI) may be cited. The average daily excretions of allantoin and uric acid by Rabbit 7 on a daily ration of 300 gm. of carrots were 42 and 9.1 mg. respectively. A change of diet from carrots to one of oats and water resulted in the daily elimination of more than twice as much allantoin. Stransky (3) has obtained results similar to these and has concluded that the smaller excretion of allantoin on the carrot diet, as contrasted to that on the oat diet, was due to the higher content of calcium and magnesium in the former diet. In view of the work of various investigators (9) relative to the increased excretion of uric acid by man, following an increased protein intake, it seems probable that the increased allantoin excretion of the rabbits just discussed might be similarly explained. The views of Stransky in regard to the influence of the calcium and magnesium content of the diet on allantoin excretion will be discussed later.

Further illustrations of the effect of diet on allantoin excretion may be seen in Table II (Periods II and III). The replacement of the carrots by cabbage in the diet of Rabbit 9 was accompanied by an increase in the excretion of allantoin from 45 to 94 mg. per day. It is to be noted (Table II, Period V) that the continuation of the oat and cabbage diet eventually led to the excretion of very small amounts of both uric acid and allantoin.

That it was the change of diet which lead to an increased excretion of allantoin, rather than a specific effect of the cabbage, was demonstrated in experiments on two other rabbits. One of these rabbits (Rabbit 8) had been on a diet of 70 gm. of oats and 100 gm. of cabbage for a period of 2 months. The average daily allantoin excretion at the close of this period was 69 mg. The cabbage of the diet was now replaced by an equal weight of carrots, and after 3 weeks on this diet, the allantoin excretion had increased to 95 mg. per day. A second rabbit (Rabbit 13) had been restricted to a diet of oats and cabbage for a period of nearly 7 months. The average daily excretions of uric acid and allantoin at the close of this period were 6.1 and 51 mg. respectively. The corresponding figures for the urines collected 8 days after the substitution of an equal weight of carrots for the cabbage were 9.9 mg. and 89 mg.

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Previously in this paper attention was called to the work of Stransky (3), relative to the excretion of allantoin by rabbits on a diet of oats as compared to a diet of fresh carrots. It was his conclusion, that the lower excretion of allantoin observed on the carrot diet was due to its higher content of calcium and magnesium. In a second paper, Stransky (3) has presented evidence that rabbits on a diet of oats and mineral water excreted less allantoin than on a diet of oats and tap water. There is no doubt that in these experiments there was a decided decrease in the excretion of allantoin during the two periods of mineral water ingestion. There was not, however, an increase in allantoin excretion during the fourth period, when the animals were again given oats and tap water. Lubienecki (10) has studied the effect of the ingestion of calcium salts upon the purine metabolism of the rabbit, the dog, and man. In five experiments with rabbits calcium chloride was administered by mouth, subcutaneously, and intravenously. In all of these experiments there was a decreased excretion of allantoin following the calcium chloride administration. In the writer's opinion these experiments are not convincing since they were so poorly controlled. Starkenstein (2) has reported one experiment in which a fasted rabbit received 5 gm. of calcium chloride *per os*. A marked decrease in allantoin excretion was observed following the calcium chloride administration and on the basis of this experiment and the work of Lubienecki, Starkenstein has stated that the ingestion of calcium salts depressed the allantoin excretion of rabbits. A more interesting phase of this question has been the attempt to explain the beneficial effects of the ingestion of mineral waters by man as a direct influence of the inorganic salts upon purine metabolism (11).

To obtain further data relative to the effect of an increase in the calcium content of the diet upon allantoin excretion, Rabbit 7 (Periods VI and VII, Table I) was given a diet of oats and milk to replace the diet of oats and distilled water of the previous period. The average excretion of allantoin for the first 3 days of Period VII was 44 mg. in comparison to an average excretion of 100 mg. for the previous period. It is interesting to note that the allantoin excretion on the diet of 40 gm. of oats and 100 cc. of milk was practically the same as that observed when the rabbit consumed 300 gm. of carrots per day (Period V). This seems to

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be a confirmation of the theory that diets high in calcium salts lower the excretion of allantoin. It should be remembered, however, that the allantoin excretion for Period III (51 mg.) was nearly as low as that of Period VII (44 mg.), although the calcium content of 100 gm. of cabbage is less than one-half of that of 100 cc. of milk. The allantoin excretion of the last 3 days of Period VII was not decreased by the administration of additional calcium chloride, but there was on the contrary a slight increase in allantoin excretion for the last 2 days. The experiment was discontinued because the animal had developed a very severe diarrhea, which made the collection of urine impossible.

A similar experiment was made with Rabbit 9 (Table II, Periods V and VI). In the case of this rabbit, an increase in the calcium of the diet, due to the feeding of milk, was accompanied by an increased excretion of allantoin. It is to be noted, however, that in the case of Rabbit 7, the milk was given after a period in which the purine excretion was at a relatively high level for this rabbit, while in the case of Rabbit 9, the milk feeding followed a period of low purine excretion. A further study of the effect of the ingestion of calcium salts upon the allantoin excretion of the rabbit was made with Rabbits 11, 14, and 15. The daily diet of Rabbit 11, consisting of 70 gm. of oats and distilled water *ad libitum*, was modified by the addition of 100 cc. of milk. The average daily allantoin excretion for the period of milk consumption (8 days) was 35 mg. as compared to 52 mg. for the earlier period. The addition of 1 gm. of extra calcium chloride to the milk for several days was without effect upon the allantoin excretion. Rabbits 14 and 15, after 1 month on a diet of oats, cracked corn, carrots, and cabbage were given 2 and 3 gm. of calcium chloride by stomach tube on successive days. The allantoin excretion of Rabbit 14 was increased approximately 30 per cent on the 2 days of calcium chloride administration, while that of Rabbit 15 remained unchanged.

In summary then, a decrease in the excretion of allantoin was noted in two of three rabbits, following the feeding of milk, a calcium-rich food. In no case, however, did the administration of calcium chloride lead to a decrease as previously reported by Lubienecki (10) and Starkenstein (2).

Whether the high level of allantoin and uric acid excretion of

the rabbits, when they were first brought from the animal house was due to the previous consumption of large amounts of food, or whether it was due to the high purine content of some foodstuff in the diet of the stock rabbits is open to question. The diet of Rabbits 14 and 15 was changed from one of corn, oats, cabbage, and carrots to a diet in which the cabbage and carrots were replaced by alfalfa hay. After 20 days of this diet, the urines were again collected and analyzed. The allantoin and uric acid excretions of this latter period were double those of the former. Vickery and Leavenworth (12) have determined the adenine content of alfalfa hay and found it to be 0.066 per cent. From these data it can be estimated that approximately one-half of the increased allantoin excretion of these rabbits could be accounted for by this preformed purine in the diet. There still remains, however, a considerable amount of allantoin and uric acid, which must have another origin.

One experiment should be mentioned, in which it was demonstrated that an increased food intake was accompanied by an increased purine excretion. The average daily allantoin excretion of Rabbit 13, after 1 month on a diet of 70 gm. of oats and 100 gm. of cabbage per day was 63 mg. For the following 2 weeks the oats of the diet were increased from 70 to 100 gm. per day. The allantoin excretion at the close of this period averaged 104 mg. per day. The oats of the diet were again restricted to 70 gm. per day for a period of 1 month, and it was found that the average daily excretion of allantoin at the end of this period had decreased to 51 mg. per day. The variation in the allantoin excretion due to the addition and removal of 30 gm. of oats cannot be explained by the preformed purines in 30 gm. of oats, since Stransky (3) has found that there are only 18.5 mg. of purine nitrogen in 100 gm. of oats. It is possible that the effect of an increased oat consumption upon the allantoin excretion of a rabbit, observed in this experiment, is similar to the effect of an increased protein consumption upon the uric acid excretion of man. The increased allantoin excretion of Rabbit 7 (Table I, Periods V and VI), which was observed in changing the diet from carrots to oats and distilled water, might also be explained on this basis, rather than on the change of the calcium content of the diet as proposed by Stransky. Langfeldt and Holmsen (13) have reported that dogs on a high

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meat diet excreted amounts of allantoin and uric acid which were greatly in excess of that which might be attributed to the purine content of the meat.

In the paper of Langfeldt and Holmsen referred to above, an extensive study of the purine excretion of dogs has been made. These authors have pointed out that the most striking feature of their work was the individual variation of their dogs with respect to purine excretion. A similar variation has been observed in rabbits with respect to their purine excretion. The allantoin excretion of individual rabbits, as determined immediately after the animal was brought to the laboratory from the animal house, varied from 75 to 250 mg. per day, although all of these rabbits had access to the same diet. That these individual variations continued after confinement was observed in several experiments in which rabbits were brought to the laboratory at the same time, and placed in metabolism cages on similar diets. If, for example, the allantoin and uric acid excretions of one rabbit were twice as great as those of a second rabbit for the initial period of caging, this variation would persist, although the purine excretion of both rabbits may have decreased to one-half during the period of caging.

The uricolytic indices of the rabbits have been calculated for the various periods of study and have been found to be remarkably constant. The average uricolytic index of the entire group (ten rabbits) was approximately 91. The greatest variation from this value was found to be 81 for Rabbit 9 (Period II) and 83 for Rabbit 7 (Period V). The uricolytic index of Rabbit 13 computed for nine periods over a period of $7\frac{1}{2}$ months varied between 90 and 92.

Although many of the findings presented in the present paper cannot be satisfactorily explained, it was thought advisable to present them at this time, with the hope that the correlation of the present data with those of other workers, might lead to a solution of some of the problems involved in purine metabolism. In view of the remarkable decreases in the excretion of allantoin and uric acid, when the rabbit is caged on a restricted diet, one should be extremely cautious in the interpretation of experimental work in which this factor has not been recognized.

SUMMARY.

The daily allantoin excretion of the rabbit is subject to wide variations. The most striking variation is the decreased excretion of allantoin which is observed when the rabbit is caged over a long period of time on a constant diet. A marked change in the allantoin excretion may be obtained by a change in diet, which cannot be explained by the preformed purines of the diet. The administration of calcium chloride *per os* produced an increase in the excretion of allantoin, rather than a decrease as reported by other investigators. However, an increase in the calcium of the diet, obtained by feeding milk, was followed by a decreased excretion of allantoin in several experiments. The uricolytic index of the rabbit was found to be approximately 91.

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THE PREPARATION OF *d*-GALACTURONIC ACID FROM LEMON PECTIC ACID.*

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INTRODUCTION.

A noteworthy advance in the knowledge of the constitution of the pectic compounds was afforded through the excellent researches of Ehrlich and his coworkers (1), who discovered that *d*-galacturonic acid, an isomer of *d*-glucuronic acid, was a constituent of all pectic substances so far examined. Later researches showed that the pectic acid obtainable from sugar beet pectin (2) can be regarded as a triacetyl-arabino-galacto-dimethoxy-tetragalacturonic acid, wherein the galacturonic acid comprises approximately 68 per cent of the molecule. Essentially the same structure was deduced for the pectic acid from flax pectin (3) which is made up of 61 per cent of galacturonic acid, and the pectic acid from orange peelings which is composed of 73.7 per cent of galacturonic acid.

In 1924, the senior writer had occasion to require considerable quantities of galacturonic acid for biochemical studies conducted in conjunction with Dr. James G. Dickson of the Department of Plant Pathology. After considerable experimentation with the various commercial preparations obtainable and several prepared in the laboratory from sugar beet pulp and apple pumice, it was found that a lemon pectin preparation from the California Fruit Growers Exchange Laboratory afforded the most economical and convenient starting material. In view of the fact that the study of the chemistry of the pectin constituents (4) has recently attained a rather preeminent position in organic and plant biochemical

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researches,[1] which has created a considerable demand for both galacturonic and glucuronic acid, it is deemed advisable to publish the method of isolating galacturonic acid which has been used with remarkable success in our laboratory.

The lemon pectin preparation obtainable from the California Fruit Growers Exchange, a grayish white amorphous powder completely soluble in hot water, is prepared by extracting the lemon pulp with citric acid at a temperature of about 90°. After concentrating, the pectin is precipitated with alcohol, the precipitate pressed and washed with alcohol, again pressed, and finally dried. The amount of impurity present is very small. This pectin preparation is perhaps the purest commercial preparation obtainable. It is supplied at such a nominal cost that the lengthy and tedious process of preparing the pectin on a large scale in the laboratory after the procedure of Ehrlich (2) proves to be a very expensive and time-consuming process.

The lemon pectin preparation which we have used is in reality lemon pectic acid after the nomenclature of Ehrlich (1). It contains approximately 80 per cent of galacturonic anhydride. It is therefore 8 to 10 per cent higher in this constituent than the pectic acids obtained by Ehrlich from other sources. This difference is readily accounted for through the researches of Ehrlich and Kasurahly ((5) p. 174, 175). Apparently in the lemon pulp, either by the action of organic acids or through enzymatic action a partial hydrolysis of the complex pectin molecule proceeds before the tissue is killed. This results in the removal of some of the carbohydrate constituents (arabinose and galactose). Or in the process of isolating the pectic acid from the pulp by means of citric acid at 90°, a partial removal of the readily hydrolyzable carbohydrate constituents is brought about. The complex galac-

[1] Recently uronic acid derivatives have through the work of Heidelberger, Goebel, and others obtained an unexpected and important biochemical significance. Aldobionic acids compose specific polysaccharide substances produced by Type III pneumococcus and Type A Friedländer bacillus, and are also constituents of the plant gums. Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, **74,** 613 (1927). Goebel, W. F., *J. Biol. Chem.*, **74,** 619 (1927). Heidelberger, M., and Kendall, F., *J. Biol. Chem.*, **84,** 639 (1929). Butler, C. L., and Cretcher, L. H., *J. Am. Chem. Soc.*, **51,** 1579 (1929). Norman, A. G., *Biochem. J.*, **23,** 524 (1929).

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turonic acid ester however is so stable that it is unaffected in either one of the processes mentioned. For the preparation of galacturonic acid from lemon pectic acid the high galacturonic acid anhydride content is obviously advantageous since thereby the yield of the free galacturonic acid is appreciably increased.

A survey of the work done in the last decade on the polyuronic acid anhydrides and also the recent work on the aldobionic acids, reveals a significant fact with reference to the properties of the free uronic acids. Whenever the experimental procedure used in the isolation of a uronic acid involves the use of weak mineral acids as the hydrolytic agent, the actual yield of the specific uronic acid isolated is invariably extremely low, in most cases less than 30 per cent of the theoretical quantity obtainable (calculated on the basis of the uronic acid content of the source material).

The complex polyuronides are quite stable towards weak mineral acids, and hydrolyze very slowly. The reverse is the case with the free uronic acids. They are readily decarboxylated by weak mineral acids (2 to 5 per cent strength) resulting in the formation of carbon dioxide, furfuraldehyde, and furan substances. We have found that it is the instability of the freshly liberated uronic acids in weak mineral acid solution that is primarily responsible for the low yields obtained. In this paper we have accounted for the quantity of galacturonic acid destroyed under our experimental conditions. In a forthcoming paper we shall publish detailed results on the stability of *d*-galacturonic acid and *d*-glucuronic acid in weak mineral acid solutions.

The method of preparing *d*-galacturonic acid through its barium salt from lemon pectic acid, which is outlined in detail below, surpasses in expediency and efficiency all methods hitherto published by Ehrlich and other workers, wherein various other fruit and plant tissues serve as the starting material (6). Incidentally, this appears to be the first time that *d*-galacturonic acid is reported to be the basic constituent of lemon pectin, thereby confirming Ehrlich's view that galacturonic acid is the fundamental constituent of all pectic substances. It should however be stated, that Suarez (7) was the first to isolate *d*-galacturonic acid from lemon pulp, in the form of its barium salt.

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EXPERIMENTAL.

Preparation of the Barium Salt of d-Galacturonic Acid.

The hydrolysis of the pectin is accomplished in 2.5 per cent sulfuric acid (using 15 gm. of the dried pectin in a liter of the acid) by boiling under a reflux condenser for 15 hours. The acid is neutralized with a suspension of barium carbonate in water while the contents of the flask are maintained at 90° by heating on a water bath. After the neutralization is completed the somewhat darkened solution is cleared with blood charcoal by heating for an additional 15 minutes. The barium salts and the charcoal are removed by filtering through a Buchner funnel and washed with hot water. A second clarification is usually necessary and is performed by heating the solution for 15 minutes with more charcoal and some infusorial earth. The solution is then filtered through a Buchner funnel containing a mat of paper pulp on a hardened filter paper. Thereby the fine charcoal which would later be precipitated with the barium galacturonate is completely removed. The solution is then concentrated to 0.1 of its original volume at 50° under 15 mm. pressure. After removing any flocculent matter present, the solution is cooled and poured into 4 volumes of 95 per cent alcohol. This procedure is accompanied with vigorous stirring to prevent the barium galacturonate from settling out in large masses. The salt is filtered off and washed with hot 95 per cent alcohol and ether and is then redissolved in 100 cc. of hot water. It is advisable to clarify the aqueous solution again with a small portion of infusorial earth before reprecipitating it with alcohol, for the purer the barium salt of galacturonic acid is, the more slowly it precipitates from an alcoholic solution. After the second precipitation the barium galacturonate is partially dehydrated by suspending first in 95 per cent and then in 99 per cent alcohol. The alcohol is in turn replaced with ether. The ether is then removed by spreading the salt on a porous plate in a vacuum desiccator over calcium chloride. After the preliminary drying mentioned above the barium galacturonate is completely dehydrated at 80° in a vacuum tube or oven containing P_2O_5. The salt should emerge from this drying process as a snow-white amorphous powder.

A barium determination made by directly ashing a small quan-

tity in a crucible with concentrated sulfuric acid serves as a reliable criterion of the purity of the salt. Preparations with a barium content of 26 to 27 per cent are readily obtained. Theory demands for $(C_6H_8O_7)_2$ Ba. 26.3 per cent barium. The yield of the barium salt from 80 gm. of pectin with an initial uronic anhydride content of 80 per cent is approximately 40 gm. This is equivalent to 42.6 per cent of the theoretical quantity obtainable. Approximately 32 per cent of the theoretical amount obtainable as the barium salt is destroyed in the course of the 15 hour hydrolysis by the 2.5 per cent sulfuric acid, a loss that cannot be minimized.[2] The remaining 27 per cent theoretically obtainable as the barium salt is lost in the processes of purification and through the formation of reversion products. When uronic acids are heated in weak mineral acids some condensation and reversion products are formed which in turn are not rehydrolyzed to the free acid.

Conversion of Barium Galacturonate into d-Galacturonic Acid.

10 gm. of the dried barium salt are dissolved in 400 cc. of water and decomposed with 0.1 N sulfuric acid, slightly less acid than is necessary to decompose completely all of the salt being used. It is necessary to prevent an excess of acidity due to sulfuric acid for the production of reversion products and lactone formation is accelerated in acid solution. The decomposition is best brought about by admitting the 0.1 N sulfuric acid from a burette while the solution containing the barium salt is being agitated with a mechanical stirrer. After the acid has been added the solution is

[2] This loss was quantitatively ascertained by the following control experiment. Two 1.0 gm. samples of the pectin were boiled for 15 hours in 2.5 per cent sulfuric acid in the apparatus recently described by us for the determination of uronic acids (see Dickson, A. D., Otterson, H., and Link, K. P., *J. Am. Chem. Soc.*, **52**, 775 (1930)). The quantity of carbon dioxide liberated was 0.0673 gm. and 0.0682 gm. respectively, which is equivalent to 6.73 and 6.82 per cent of carbon dioxide. The carbon dioxide values multiplied by the factor 4.4 for the conversion into uronic acid give 29.6 and 30 per cent of galacturonic acid. Calculated on the basis of the actual amount of galacturonic acid that a pectin sample containing 80 per cent of galacturonic anhydride would yield, if the hydrolysis were quantitative, a destruction of 29.6 and 30 per cent of the pectin molecule is equivalent to a destruction of 33.6 and 34.1 per cent of the total amount of galacturonic acid initially present. (We wish to thank Mr. Carl Niemann for conducting the control experiments.)

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warmed to 35° and maintained at this temperature for 30 minutes to accelerate the settling of the barium sulfate in a form to permit filtering. The barium sulfate is removed and the solution carefully concentrated at 40° under reduced pressure to a syrup of the consistency of glycerol. The thin syrup is poured into sufficient 95 per cent alcohol to insure a final concentration of above 90 per cent after dilution by the water in the syrup. The undecomposed barium galacturonate will fall out as a flocculent precipitate, at the same time any inorganic barium will also be precipitated. After these foreign substances are removed, the alcoholic solution is concentrated to a syrup which is taken up in hot 92 per cent alcohol, filtered from any flocculent material that exists, and decolorized with a small amount of blood charcoal and a pinch of infusorial earth. The alcoholic solution is then finally concentrated to a syrup which will still permit an easy transference to a round bottom crystallizing dish. By keeping the dish in a desiccator for 24 hours over calcium chloride, crystallization usually begins after the addition of a few cc. of absolute alcohol and is complete after 24 hours. The crystals are filtered, washed with 95 per cent alcohol, then with absolute alcohol, and finally with ether. The drying should be conducted at room temperature over P_2O_5 in a vacuum desiccator or vacuum tube. *Heating discolors the free acid and eventually destroys it.* The galacturonic acid crystallizes in microscopic needles with a melting point of 159–160°. The acid usually becomes yellow at 115°, red at about 135°, dark brown above 150°, and melts at 159° with frothing and decomposition. The optical rotation in water after 24 hours is +53.59°.

The carboxyl group of galacturonic acid can be directly titrated with phenolphthalein as the indicator. The neutralization value can therefore be used as an accurate criterion of purity. The direct neutralization of 0.5560 gm. of *d*-galacturonic acid required 26.10 cc. of 0.1 N NaOH. Upon this basis 1.0 gm. would require 46.95 cc. of 0.1 N NaOH, whereas theoretically 1.0 gm. of $C_5H_{11}O_5$-(COOH) requires 47.20 cc. of 0.1 N NaOH.

The yield of pure *d*-galacturonic acid readily obtainable from 10.0 gm. of the barium salt is approximately 2.21 gm., equivalent to 30.5 per cent (theoretical from 10.0 gm. of barium salt is 7.44). By recrystallizing the mother liquors it is possible to increase the yield to 50 per cent.

SUMMARY.

A convenient source material for the preparation of crystalline *d*-galacturonic acid is lemon pectic acid which can be obtained commercially. The pectic acid is hydrolyzed in 2.5 per cent sulfuric acid and the galacturonic acid first obtained as its barium salt. The barium galacturonate is converted into the free galacturonic acid by decomposition with 0.1 N sulfuric acid. Precise directions for the preparation of both compounds are given.

In conclusion the writers wish to express their gratitude to Mr. W. E. Baier and Mr. C. O. Wilson of the Research Department of the California Fruit Growers Exchange, Ontario, California, who kindly furnished the lemon pectin preparations used in this research.

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XUM

THE PREPARATION OF THE CRYSTALLINE OVARIAN HORMONE FROM THE URINE OF PREGNANT WOMEN.*

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(Received for publication, February 1, 1930.)

Owing to the number of inquiries received concerning the preparation of purified extracts of the ovarian hormone, the data, which may be of service to other investigators, are being published even though certain phases of the work are still incomplete. The procedures described have been useful in the isolation of the pure compound but have now been replaced by methods which are adapted to quantity production. Following the isolation of the crystalline hormone, it was desirable to obtain large quantities of the crystals in order that we might study their chemical constitution.

Extraction of the Hormone from Urine.

In a preliminary note, we (Veler and Doisy, 1928) published data on the extraction of urine with ethyl ether. Subsequently we have tried to find suitable ways of avoiding the use of this solvent because of the fire hazard and because of its tendency to form peroxides which may cause serious losses (Thayer, Jordan, and Doisy, 1928) of the hormone.

Another extraction procedure based upon the well known solubility of the hormone in oils has been used. Though the procedure is more time-consuming than the use of the continuous liquid extractor after the apparatus of the latter is set up and operating, it is believed that a number of workers may be glad to

* An abstract of this paper was presented before The Thirteenth International Physiological Congress at Boston, August, 1929. Lantern slides of Figs. 1 and 2 were exhibited at the Congress.

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obtain a plentiful supply of the hormone with so little expenditure of labor as is involved in stirring a sample of acidified urine with olive oil. From a liter of urine we have obtained 700 rat units of hormone by agitating with a motor-driven stirrer with 50 cc. of olive oil for 20 or 30 minutes. The operation can be conducted in a separatory funnel and the urine tapped off, leaving an olive oil solution of the hormone which will suffice for many purposes. We have found that while the olive oil extraction is not useful for quantity production, it is quite satisfactory for the preparation of small quantities for physiological experiments. It may be added that our information regarding purification was first worked out on these olive oil extracts, as they have seemed to be easier to purify than extracts made with other solvents. Subsequently, our procedure has been modified to take care of the quantity production by the continuous liquid extraction apparatus which will be discussed in detail in a later paper.

Our experience indicates that it is best to acidify the urine with HCl to pH 4.0 and allow uric acid to settle out. If the urine has been allowed to undergo ammoniacal fermentation, the calcium phosphate precipitate causes trouble and the oil shows a tendency to emulsify in the alkaline urine.

An additional reason for acidification is the much greater yield that is usually obtained if the urine is strongly acidified some days prior to the actual extraction. For example, with Urine 83, which was rather strongly alkaline, about 75 per cent of the hormone was left in the urine after the extraction with oil; after acidification to Congo red paper and with the same extraction process, less than 25 per cent was left in the urine.

Though we have not investigated the point in detail, we have not detected loss of the hormone due to unsatisfactory preservation of the urine. As we have made collections only once weekly, in many instances the chloroform has evaporated and the urine has a decidedly foul odor. Nevertheless, there seems to be little difference in the amount of hormone recovered from such urine samples and those which have been well preserved. Furthermore, we have obtained good yields from urine samples which have remained in the laboratory for months.

1 liter of Urine 62, acidified with HCl, was stirred for 30 minutes with 5 successive 50 cc. volumes of olive oil. The first ex-

tract contained 714 rat units, the second 143, the third 125, the fourth 125, and the fifth 143, a total of 1250 rat units. The total obtainable by butyl alcohol extraction of this urine was 2200 rat units per liter. Urine 63, treated similarly, gave in the successive extracts 1250, 167, 250, 250, and 250 rat units. The irregularities in the values may be due to errors of the biological assays or to insufficient time for equilibrium between the two phases. It seems from the data cited that this extraction does not follow a simple distribution and one would conclude that unless the supply of urine is limited, economy of time would indicate the practice of a single extraction.

TABLE I.

Extraction of Hormone from Olive Oil with Ethyl Alcohol.

Preparation No.	Before extraction.	After two extractions with 500 cc. alcohol.	After two additional extractions with 200 cc. alcohol.
	rat units	*rat units*	*rat units*
33	3,333	>800 <1000	<170
331	5,000	>1000 <1700	<400
332	5,000	<666 >833	>200 <333
333	16,666	>555	<200

6 liters of Urine 61 were extracted with 5 100 cc. volumes of olive oil, the yield of the combined oil extracts being 4060 rat units. Urine 67, extracted with 6 100 cc. volumes, gave 3340 rat units.

Purification of Crude Extracts.

The olive oil extracts injected into rats produced no outward manifestation of ill effects. However, for our purpose it was desirable to obtain aqueous solutions of the hormone. The next step is to remove the hormone from the oil. This is readily accomplished by shaking the oil with hot 95 per cent ethyl alcohol in a separatory funnel. In the experiments listed in Table I, the volume of the oil was 500 cc.

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It is apparent from Table I that the extraction of the hormone from the oil can be accomplished with ease. For example with Preparation 33 containing 3333 rat units in 500 cc. of oil, after the four extractions with hot alcohol less than 170 rat units or 2 per cent of the hormone remained in the oil. On attempting to apply the procedure used with the alcoholic extracts of liquor folliculi (Thayer, Jordan, and Doisy, 1928) we rarely obtained aqueous solutions that were satisfactory with regard to purity or efficiency of recovery. After about twenty-five failures, even though several slight modifications had been introduced in the hope of perfecting the method, we gave it up and started again from the beginning. Many different solvents were tried in various orders and the described procedure is the result of our failures and successes. For example, in the "pe 1" step (see next paragraph) if ethyl ether is used instead of butyl alcohol, from 20 to 40 per cent of the hormone is precipitated and does not appear in the final aqueous solutions. In our hands the procedure finally adopted yields a high percentage of the hormone in the form of a clear, colorless, potent aqueous solution.

The alcoholic solution obtained from the olive oil extract of 12 liters of urine is concentrated to about 75 cc. and poured into a separatory funnel; 25 cc. of water are added, the solution is acidified with HCl to Congo red paper, and the oil removed by two or three extractions with 20 cc. portions of petroleum ether (Preparation 75 pe). The alcoholic solution is distilled to dryness, the residue taken up in 100 cc. of butyl alcohol which is extracted three times with 20 cc. of dilute NaOH and once with dilute HCl (aqueous solution is Preparation 753). The butyl alcohol is distilled and the residue leached with ethyl ether (peroxide-free); frequently we add a little ethyl alcohol to dissolve the residue and again distil to dryness between the leachings with ether. Four or five leachings suffice. The ether extract is centrifuged, and the solution (residues insoluble in ether labelled Preparation 75 a) poured off and distilled; this residue is dissolved in 10 cc. of butyl alcohol, transferred to a centrifuge tube, and 5 volumes of petroleum ether added. After being chilled in the refrigerator, the tube is centrifuged and the supernatant fluid poured (the residue is Preparation 75 pe 1) into a separatory funnel of appropriate size. Generally we have shaken this petroleum ether-butyl alcohol solution with 2 50 cc.

volumes of dilute HCl (Preparation 75 c), then added 50 cc. of petroleum ether, and shaken again twice, added 50 cc. more, and repeated the extraction. These combined acidic aqueous solutions (Preparation 751 c) contain a considerable amount of fairly pure hormone, but a larger percentage can then be recovered by shaking the butyl alcohol-petroleum ether solution with a few portions of dilute NaOH (Preparation 752 c). This alkaline solution is grossly impure but can readily be purified, as will be described later. In checking up on the yields and losses, the petroleum ether solution is tested (Preparation 75 res) for activity.

TABLE II.

Purification of Olive Oil Extracts of Urine.

Preparation No.	Discarded fractions.		Preparation No.	Retained fractions.		
	Weight.	Activity.		Weight.	Activity.	
					Total rat units.	Weight of 1 rat unit.
	mg.	*rat units*		*mg.*		*mg.*
75 pe		>171 <400	75 c		616	
753	1044	>300 <400	751 c	36	2440	0.015
75 a	634	>407 <610	752 c	360	6000	0.06
75 pe 1	Trace.	Discarded.				
75 res	168	>350 <467				
Total.....	1846	1228		396	9056	

In Table II we give a summary of the distribution of solids and the hormone. The weight of Preparation 75 pe was not determined because of the large amount of olive oil that this fraction always contains. The work is not so time-consuming as the large number of fractions would indicate. Certain steps may be simplified, but until our experience with the alterations is more adequate, it is impossible to say whether the contemplated changes are advantageous. In the meantime, a method which will easily lead to purified extracts of the hormone or even the crystals is available.

The next procedure is the purification of the dilute acidic and basic solutions (*i.e.* Preparations 751 c and 752 c, Table II). Since both are treated in somewhat the same way and the activity of the alkaline solution is generally much greater, we will describe in detail only the one process.

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Several batches of the alkaline solution were mixed and the resulting solution (volume 550 cc.) extracted with 6 200 cc. volumes of ethyl ether. The combined ether solutions were washed with a little dilute NaOH, dilute HCl, and water, then distilled to dryness. The residue was dissolved in 5 cc. of butyl alcohol, 25 cc. of petroleum ether added, and the mixture cooled and centrifuged. The precipitate was washed once with 10 cc. of petroleum ether and a sample of the solution taken for the determination of solids (82 mg.) and activity (15,000 rat units). The butyl alcohol-petroleum ether solution was washed twice with a small volume of dilute HCl and the aqueous solution discarded, 40 cc. of petroleum ether were added, and the solution extracted twice with 50 cc. of dilute HCl; 25 cc. more of petroleum ether were added and the extraction with 50 cc. of dilute HCl carried out four times. The six aqueous extracts were combined and this solution assayed 6600 rat units of a potency exceeding 1000 rat units per mg. A large amount (9200 rat units) of the hormone still in the petroleum ether was recovered by extraction with dilute NaOH and the process of purification described in this paragraph repeated.

The aqueous solution containing over 1000 rat units per mg. was combined with another of similar potency and extracted with ethyl ether, the ether distilled, the hot flask evacuated, and the dry residue leached with anhydrous ether. This solution was centrifuged to remove a small quantity of suspended matter, transferred to a flask, distilled, and the flask freed from ether vapors. The flask was cooled and crystals formed. These crystals were washed with cold petroleum ether twice, dissolved in ethyl alcohol, and their weight determined. They were purified by distilling the alcohol, leaching the residue with small volumes of dilute NaOH, and then extracting the alkaline solution with ethyl ether. Upon distilling the ether, leafy crystals (weight 1.39 mg.) of the type shown in Fig. 1 were obtained. These crystals were dissolved in 3 cc. of ethyl alcohol and precipitated with distilled water. The crystalline shape in this case was quite different (Fig. 2) but the physiological assay showed these crystals to be exceedingly active.

Since this first crystalline product was obtained, we have found it easy to secure the crystals; in fact, every preparation of sufficient purity has been converted into the flat platelets with the rhombohedral shape. Occasionally upon recrystallization of the flat plates,

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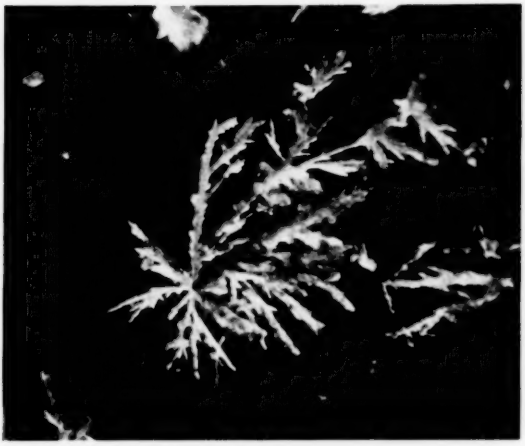

FIG. 1. × 16. Preparation 115 pe 2. It was subsequently converted to the form shown in Fig. 2. The sample was photographed in a distilling flask; owing to the curvature the focus is not perfect.

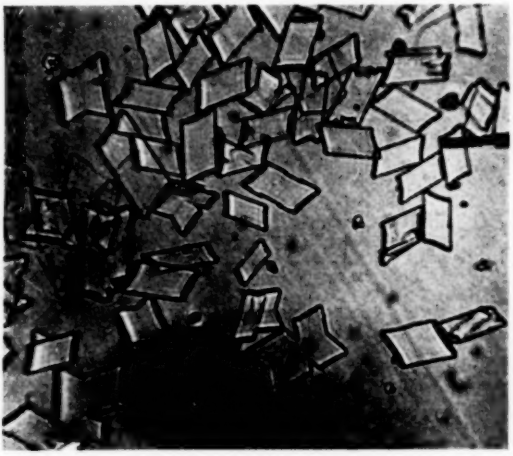

FIG. 2. × 416. Preparation 1141. Reproduction of the first preparation of platelet crystals.

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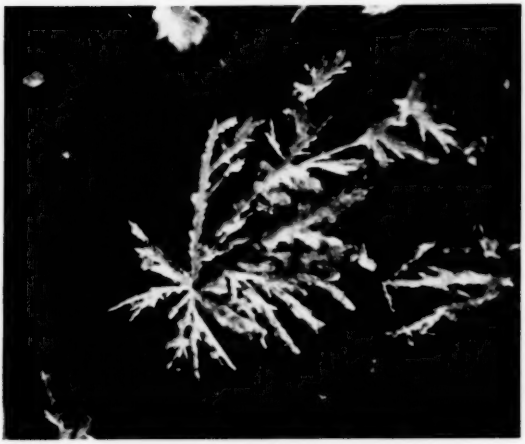

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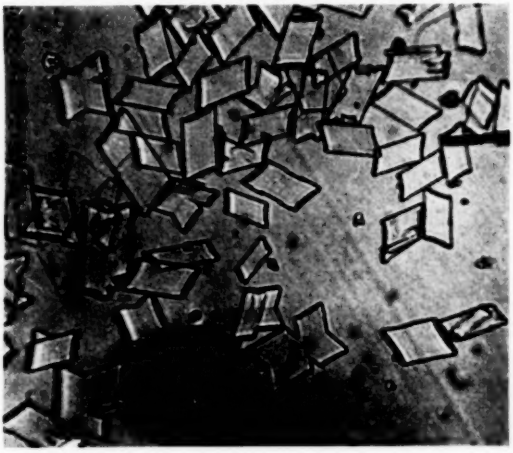

FIG. 2. × 416. Preparation 1141. Reproduction of the first preparation of platelet crystals.

they seem to form solid figures of considerable thickness which appear to be made up of individual plates of various sizes, as illustrated by Fig. 3. Of course many modifications have been introduced, including a large scale extraction procedure and as soon as the real improvements can be separated from the less successful, full details will be published. A word regarding the two crystalline forms might be in order. The leaflet type frequently appears when a solution is distilled to dryness, whereas the platelet form

FIG. 3. × 30. Photograph showing the formation of thick crystals from the rhombohedral plates. The crystal seems to be built up of the typical plates of sizes increasing from the apex to base.

usually is obtained when a solution in ethyl alcohol or acetone is precipitated with water. One of our early preparations appeared to be uncontaminated with oil or any other substance. It existed entirely in the leaflet form in so far as we could determine by microscopic examination. Upon crystallization from ethyl alcohol by the addition of hot water and subsequent cooling, we obtained only the platelet form. Careful microscopic examination of many fields revealed only platelets.

At The Thirteenth International Physiological Congress, lan-

tern slides were shown which gave in detail the procedures that have just been described and reproductions of the two crystalline forms were exhibited. Owing to the inherent difficulties, the actual crystals were shown to only a number of people who are interested in this field. The evidence in favor of the view that the crystals were in reality the hormone was stated and perhaps might be repeated here. (1) The potency of the crystals exceeded the potency of any non-crystalline product that had been studied by ourselves or other investigators.[1] (2) Several preparations of the platelet crystals had the same potency. (3) Careful microscopic examination revealed only one kind of crystal. It seemed very unlikely that a contaminating substance could be present except as mixed crystals unless it was present in very small amount. (4) Repeated recrystallization did not diminish the potency and the mother liquors tested were less active than the crystals.

Unfortunately we were unable to give the exact potency of the crystals, as our supply of test animals during the summer was too limited. We were able to state with certainty that the potency exceeded 2000 rat units per mg. Our assays, which have been completed with a large number of rats, show the potency to be at least 3000 rat units per mg. Values intermediate between 3000 and 4000 rat units were not tested. Results in a large number of the rats were positive with the 4000 units per mg. dosage, but the requisite number for the 75 per cent of those used was a little short. Assays of more extensively recrystallized preparations give a similar unitage.

The potency of the crystals will vary within rather wide limits, depending upon the exact procedure of assay. We still adhere to

[1] Since our announcement at The Thirteenth International Physiological Congress (*Am. J. Physiol.*, **90,** 329 (1929)) Butenandt (*Naturwissenschaften*, **17,** 879 (1929)) has also announced his isolation of the crystalline hormone. In his introductory paragraph in which he acknowledges that he has heard of our announcement, he also points out that the activity of his crystals is similar to the potency of ours. Wieland, Straub, and Dorfmüller (*Z. physiol. Chem.*, **187,** 97 (1929)), have also published work on the purification of the hormone but unfortunately their photographic reproductions are so poor that it is impossible to see what their crystals look like. They state that their product is probably not a pure substance. Furthermore, it appears from the assays that their preparation is much less potent than the crystals described in this paper.

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a division of the total dosage into 3 equal volumes injected at intervals of 4 hours. 1 rat unit is the minimum quantity that will produce cornification in 75 per cent of the animals and most of the remaining 25 per cent should show evidence of the effect of the injection. For important assays we generally use twenty different animals for each volume injected.

DISCUSSION.

While we believe that our work indicates clearly that the crystals isolated from the urine of pregnant women are the follicular hormone, it is desirable to review the situation in order that the reader may not be misled.

On p. 502 we stated that the purification procedure used with extracts of liquor folliculi could not be satisfactorily applied to the hormone solutions derived from urine. This may be due to a number of factors, the most important of which are a chemical difference between the physiologically active substances from the two sources, and a difference in the nature and amount of contaminating substances to be removed. It seems to us that the latter is the probable cause. At least, one encounters many instances in this kind of work in which the presence of one substance materially influences the behavior of another toward a solvent.

It seems obvious that a word should be said about the identity of the follicular hormone with the active substance isolated from urine. In so far as the gross physiological reactions have been studied by ourselves and others, there is no difference in the response of the animals toward material from the two sources. The injection of solutions of the crystals produces the estrous response of ovariectomized rats and the opening of the vagina of immature rats, which reactions are produced by follicular extracts. However, in spite of the assumption of some investigators including ourselves that the active material from the two sources is identical, this must remain as an open question until the crystals are isolated from the ovary and compared with those from the urine. It is conceivable that a group of substances possessing similar physiological properties but differing chemically and in activity may be the result of biological reactions occurring in the body after the liberation of the hormone from the ovary. One need only

recall the extraordinary power of the body to oxidize, reduce, detoxicate, etc., and correlate this with the modern advances in chemotherapy, in which it has been demonstrated that very slight changes in the molecule may produce profound changes in the biological behavior of a compound, to realize that it would be foolhardy to maintain without reservations that the crystals from the urine are the original hormone. A very interesting situation bearing on this view has arisen with respect to the sex hormone of the anterior lobe of the pituitary. Similarity of some chemical, physical, and physiological properties does not necessarily prove identity.

Regarding the question of the identity of the crystals that we have isolated with the hormone, we can say that since our first report in Boston, we have subjected the crystals to a more rigorous set of procedures without diminishing their potency. Seven different solvents have been employed in an attempt to alter the activity of the crystals. One specimen has been recrystallized twenty times. Judged by the appearance and melting point, the final product seems to be a pure substance and yet the potency remains around 3000 rat units per mg. Since, in spite of rigorous attempts to separate the hormone from our crystals this has not been accomplished, we feel that the conclusion that the crystals are the hormone (or at least the potent substance of urine) is justified. It might be well to add that though we think our conclusions are well founded, we believe that all chemists will agree with the view of Harington and Scott (1929) that the proof is not absolute until the constitution of the compound is determined, synthesis effected, and the synthetic found to possess the same physiological activity as the natural compound.

We are glad to record our appreciation of the excellent assistance rendered by Miss Helen FitzSimmons in preparing the test animals and conducting the assays.

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THE ACTION OF NITROUS ACID ON CASEIN. II.

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When nitrous acid acts upon proteins, nitrogen is liberated and the resulting products, known as deaminized proteins, show properties differing in important respects from the original proteins from which they are derived (1). The nitrogen liberated in the reaction is usually considered to originate from the reaction between the nitrous acid and the ϵ-amino group of lysine (2). Other reactions which involve the characteristic groups of amino acids other than lysine have also been suggested. Thus, according to some investigators, tyrosine is so altered in the process of deamination that deaminized proteins give no Millon's test nor can tyrosine be isolated after hydrolysis of the protein (3). No adequate explanation has been given for the yellow or yellow-brown color characteristic of deaminized proteins.

In view of the development of improved methods of analysis of proteins within recent years, it seemed that application of these methods to the analysis of deaminized proteins might yield valuable data, data which would extend earlier work from this laboratory (1, 3).

For the study of the products of deamination, casein was selected as a convenient material since this protein is easily obtainable in a purified form and yields after deamination a product which is insoluble and hence easily purified. We have studied the properties and composition of deaminized caseins in whose preparation different temperatures were employed in the hope that at lower temperatures, the possibility of secondary reactions might be more limited. Our attention has been directed chiefly to the distribution of nitrogen as determined by the Van Slyke procedure, to the content of tyrosine and tryptophane, and to the content of the basic amino acids.

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The method used for the preparation of the deaminized casein was a modification of the procedure of Skraup, as previously outlined by Dunn and Lewis (1). All the factors except the temperature of the reaction mixture during the period of the addition of the sodium nitrite were kept as nearly constant as possible. Products of deamination at varying temperatures, 0, 20, 40, and 60°, were prepared and analyzed. After the reactions of deamination had proceeded at the temperature indicated for 1½ hours, the mixture was allowed to stand for 18 hours, in the ice box in the case of the material deaminized at 0°, and at room temperature in all other experiments.

Since it was possible that contact with the acetic acid in which the casein was dissolved might alter the molecule independently of the action of the nitrous acid, one experiment was carried out in which casein was dissolved in acetic acid and the acid subsequently neutralized with sodium hydroxide. This procedure was carried out under exactly the same conditions as were used in the preparation of the casein deaminized at 20°, an amount of sodium hydroxide equivalent to the acetic acid being added instead of the sodium nitrite. The reaction after the addition of the alkali was close to the isoelectric point of casein and the protein was precipitated. This preparation, washed and dried in the usual manner, will be designated as "denaturized" casein merely for convenience in order to distinguish it from the original untreated casein.

The color of the deaminized proteins varied with the temperature of deamination, the products obtained at lower temperatures being light yellow in color while those obtained at higher temperatures were brownish. The surface of the deaminized casein became dark when exposed to light. The yield as in earlier experiments (1) was between 90 and 95 per cent of the casein used.

Not only were the deaminized caseins analyzed, but also the original samples of casein, which were used in the preparation of the deaminized proteins. Moisture and ash determinations were made on all samples and all the analytical data reported are calculated on an ash-free moisture-free basis.

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Partition of Nitrogen by the Method of Van Slyke.

The method originally proposed by Van Slyke for the analysis of proteins by the determination of characteristic groups is subject to many errors such as those due to the destruction of cystine in the acid hydrolysis, to the incomplete precipitation of the basic amino acids by phosphotungstic acid, and to the precipitation of amino acids other than the basic amino acids by the phosphotungstic acid. However, it was believed that in the comparison of two proteins as similar in nature as casein and deaminized casein, and in the comparison of deaminized caseins prepared at various temperatures, these errors would be of approximately the same magnitude and that some indication of the effects of nitrous acid on the protein molecule would be given.

Eight samples of deaminized casein, prepared under slightly different conditions, two of casein and one of "denaturized" casein were analyzed. Each analysis of those presented in Table I is the average of determinations carried out in triplicate. The method as proposed by Van Slyke was used without essential modification throughout except that the apparatus described by Koehler (4) was used for the determination of the arginine nitrogen of the bases.

"Denaturized" casein showed, within the limits of the experimental error of the method, the same analysis as casein, thus indicating that acetic acid alone had practically no effect on the protein molecule as far as could be determined by this method.

The total nitrogen of the deaminized casein was in all cases slightly less than that of the casein from which it was prepared as was also the total amino nitrogen after hydrolysis. There appeared to be a slight decrease of the arginine nitrogen of the bases, destruction of arginine increasing with the temperature of deamination, shown most significantly in the sample deaminized at 60° (Deaminized Casein VII, Table I). This was not unexpected since it has been shown by Sekine (5) and others (6, 7) that arginine may lose more than one-quarter (an amount corresponding to the nitrogen present as the α-amino group) of its nitrogen, when allowed to react with nitrous acid for long periods of time.

The results obtained for the histidine content of casein were much lower than those usually reported for casein by this method

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(4.24 to 6.55 per cent). There appeared to be an increase in the histidine content of most of the deaminized caseins. This increase, however, was probably within the limits of error of the method since histidine was determined indirectly and, in our hands, duplicate determination frequently showed marked variations. In a later part of this paper, in which the isolation and more direct determination of histidine are reported, it will be

TABLE I.

Analyses of Casein, Deaminized Casein, and "Denaturized" Casein.

Total N is reported in per cent of N in protein. The remainder of the figures are in terms of per cent of the total N present.

Protein analyzed	Casein II.	Deaminized casein No.*				"Denaturized" casein.
		V	VI	VII	VIII	
Total N	14.95	14.72	14.76	14.61	14.73	14.93
Amino "	76.59	71.69	69.62	69.45	69.81	76.25
Amide "	9.98	10.65	11.24	11.34	11.26	10.32
Melanin N	1.32	1.23	1.93	2.05	2.00	1.71
Total N of bases	20.63	17.42	15.49	13.00	15.63	20.81
Amino N of bases	13.84	8.93	7.84	5.76	7.04	13.87
Arginine N	7.63	8.17	7.16	6.37	7.08	7.80
Histidine N	1.72	3.54	3.31	3.70	5.04	1.64
Cystine N	0.59	1.60	1.55	1.55	0.83	0.65
Lysine N	10.70	4.12	3.47	1.39	2.68	10.73
Total N of filtrate	66.36	70.05	71.10	73.68	70.12	66.51
Amino N of filtrate	61.20	63.14	64.20	63.23	61.34	60.35
Non-amino N of filtrate	5.16	6.54	6.90	10.45	8.78	6.16
Total N recovered	98.30	99.36	98.77	100.06	99.01	99.35

* In the preparation of Deaminized Caseins V to VIII, the temperatures of the initial stage of the deamination were 0, 20, 60, and 20° respectively.

shown that deaminized casein actually contained less histidine than did the casein used in the deamination.

The lysine content of the solution of the bases from deaminized caseins was markedly decreased. With the ϵ-amino group replaced by a hydroxy group, lysine would presumably lose its basic properties, and the product of deamination corresponding to lysine would be expected to appear in the filtrate from the bases. This should result in increase in the amino nitrogen of that fraction equivalent to one-half of the lysine nitrogen.

Although in our analytical results an increase in the total nitrogen of the filtrate was evident in all cases after deamination, the greater part of this increased nitrogen was shown to be present in the non-amino fraction in most cases.

The results indicate that the destruction of lysine was a direct function of the temperature of deamination. Thus the proteins deaminized at 0° showed a lysine nitrogen content of 5.83 and 4.12 per cent of the total nitrogen; at 20°, 3.78, 3.47, and 2.68 per cent; at 40°, 3.31 per cent; and at 60°, 2.33 and 1.39 per cent. These results which do not indicate a complete destruction of lysine are probably misleading, however, since as shown in a later part of this paper, it was impossible to *isolate* lysine from Deaminized Casein VIII, which showed by the Van Slyke procedure a lysine nitrogen content of 2.68 per cent.

On the whole, the results obtained by the Van Slyke partition method closely resemble those previously obtained by one of us (1) and do not indicate that, under the experimental conditions used, the temperature of deamination played an important rôle in influencing the composition of the deaminized product.

Tyrosine and Tryptophane Content of Deaminized Casein.

Early in the study of the composition of deaminized proteins, it was observed that a part at least of the tyrosine was destroyed or modified in some way by the reagents used in deamination (3). The few quantitative studies of the tyrosine content of deaminized casein have been made by the use of the Folin-Denis colorimetric method (1) or some modification (3) and have indicated a lowered chromogenic value with the phenol reagent after deamination. Steudel and Schumann (8), in a recent paper published after the completion of the present study, have been unable to observe any change in either the tyrosine or tryptophane content of casein deaminized according to the procedure of Dunn and Lewis (1) (as determined by the method of Folin and Looney).

The possibility of reactions other than the replacement of the amino group has long been recognized. Treves and Salomone (9) because of the yellow color of their products and their reactions to certain tests for diazo compounds were led to postulate the formation of diazo derivatives. Dunn and Lewis (1) suggested nitroso derivatives of the various cyclic amino acids as a possible explanation of the pigmentation.

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Morel and Sisley have recently studied the reaction of nitrous acid on silk (10) and upon tyrosine (11) at low temperatures (below 10°), and believe that they have secured evidence of diazotization of part of the tyrosine. Evidently under the conditions employed by them, only a portion of the tyrosine was deaminized to form *p*-hydroxy-phenyllactic acid. They were unable to obtain complete diazotization of the tyrosine, a maximum of 70 per cent being reached in about 36 hours. The diazo derivatives of tyrosine and its products of deamination, unstable at ordinary temperatures, yielded on reduction (stannous chloride or sodium hydrosulfite) the corresponding 3-amino derivatives. These on treatment with nitrous acid gave the same color as was obtained by prolonged treatment of tyrosine with nitrous acid. They suggested that in the reaction of nitrous acid on silk, nitroso groups first entered the tyrosine molecule in a position ortho to the hydroxy group. These nitroso groups were then reduced to amino groups and the primary amine group thus formed was diazotized.

In our study we have used the methods for the determination of tyrosine and tryptophane described by Folin and Ciocalteu (12). Difficulty was experienced, however, in applying the modified Millon reaction to the hydrolysates of deaminized casein.

Analyses were made on three types of hydrolysates prepared as outlined below.

1. Acid Hydrolysate.—1 gm. of protein was hydrolyzed for 18 hours with 20 cc. of 20 per cent hydrochloric acid and the excess acid was removed by evaporation on the steam bath under diminished pressure, 9 cc. of 14 N sulfuric acid were added, and the solution made up to 100 cc. in a volumetric flask.

2. Barium Hydroxide Hydrolysate.—1 gm. of protein was boiled with 4 gm. of barium hydroxide and 25 cc. of water for 48 or 96 hours. To the hot solution were added 30 cc. of 20 per cent sulfuric acid and the flasks were heated in a boiling water bath for 30 to 40 minutes to remove any hydrogen sulfide present. The solution was then diluted to 100 cc. and filtered.

3. Sodium Hydroxide Hydrolysate.—1 gm. of protein was hydrolyzed as described by Folin and Ciocalteu (12).

The tyrosine and tryptophane were separated as described by Folin and Ciocalteu (12) except that only one-half the amounts

of reagents and sample specified by them were used with a correspondingly weaker standard. After the last washings of the tryptophane precipitate had been transferred to the volumetric flasks, 30 cc. of water, 2 drops of caprylic alcohol, and 25 cc. of saturated sodium carbonate solution were added in the order named to each flask and to the standard. The flasks were then shaken and 4 cc. of a 5 per cent sodium cyanide solution were added. The flasks were shaken until the precipitated mercuric oxide was dissolved and 5 cc. of the phenol reagent of Folin and Ciocalteu were added, the solutions after thorough mixing were allowed to stand 10 to 30 minutes, diluted to 100 cc., and the color comparisons made.

As already pointed out, Morel and Sisley (11) have observed that to the phenol group of tyrosine after treatment with nitrous acid, a diazo group was added and that this diazo group could be reduced to the corresponding amine. Since it seemed likely that the amino group might be less effective in the reduction of the phenol reagent of Folin than the diazo group, an attempt was made to reduce the hydrolysates and to compare the chromogenic values before and after reduction. Aliquots of the hydrolysates were reduced for 2 to 3 hours by the addition of a little zinc, the acid of the hydrolysates being sufficient to liberate hydrogen by its reaction with the zinc. It was hoped in this way that color development other than that resulting from the phenol group might be avoided. Analyses were then made on the reduced solutions, 4 cc. aliquots being used as already outlined. The original hydrolysates are designated as "unreduced" in Table II while the values obtained after treatment with zinc are presented in the column headed "reduced."

The results of the analyses of casein and deaminized casein by the modified Millon reaction of Folin and Ciocalteu are not presented in tabular form. The figure obtained for the tyrosine content of casein (6.51 per cent) is in good agreement with the values reported by Folin and Ciocalteu (12). It was impossible to obtain satisfactory results when this method was applied to the hydrolysates of deaminized casein, since the faint red color obtained could not be satisfactorily compared with the standard color, due to the yellow tinge in the hydrolysate. However, the color produced was probably less than half that obtained with

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an equal amount of casein. The color developed in the modified Millon reaction was not significantly affected by the reduction of the hydrolysates with zinc and sulfuric acid.

A comparison was made of the reaction of a number of phenols in the Millon test as ordinarily applied and as modified by Folin and Ciocalteu. Tyrosine and phenol were the only phenols of those studied which gave positive tests by the modified test, although all the others (vanillin, salicylic acid, catechol, resorcinol,

TABLE II.

Tyrosine Content of Casein and Deaminized Casein (Modified Method of Folin and Looney).

Hydrolytic agent	HCl 18 hrs.		NaOH 18 hrs.		$Ba(OH)_2$	
Treatment of hydrolysate	Unreduced.	Reduced.	Unreduced.	Reduced.	Unreduced.	Reduced.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Casein II	6.44	6.35	6.56	6.38		
Deaminized Casein V*	6.45	6.50	5.98	6.43		
" " VI	5.33	5.39	5.65	5.75		
" " VII	4.67	5.13	5.59	6.08		
" " VIII	4.73	4.70	4.91	5.53	3.80†	4.63†
" " A 64‡	4.82	5.56	5.68	6.05	4.43†	4.62†
					4.39§	4.52§

* See Table I for characterization of the various samples of deaminized casein.

† Hydrolyzed for 48 hours.

‡ This is the deaminized casein used by Dunn and Lewis (1).

§ Hydrolyzed for 96 hours.

hydroquinone, 3, 4-dioxyphenylalanine, and phloroglucinol) gave positive tests with the original Millon test. Phenol, compared with an equivalent amount of tyrosine, gave by the Folin-Ciocalteu procedure only about 70 per cent of the color developed by tyrosine. From the different behaviors of these substances in these two tests it seems that the reaction as outlined by Folin and Ciocalteu is more specific than the original Millon test and that color production is inhibited by the presence of substituent groups on the phenol nucleus.

The results obtained with the modification of the Folin-Looney method as described were more satisfactory (Table II). In the two proteins hydrolyzed with both sodium and barium hydroxide, the figures obtained with the sodium hydroxide hydrolysates were higher than those with the barium hydroxide hydrolysates, a result in accord with the work of Folin and Ciocalteu (12) who found lower values in the case of barium hydroxide hydrolysates. The hydrolysates of the deaminized caseins after reduction usually gave higher values than before reduction, especially in the case of the sodium hydroxide hydrolysates, where the values often approached those obtained for casein. However, in most cases, particularly with the deaminized caseins[1] prepared at room or higher temperatures (Deaminized Caseins VI to VIII, Table II), there appeared to be a decrease in tyrosine as compared with the values for casein.

The question is again raised as to whether this apparent change represents an actual destruction of tyrosine or the inhibition of color production by the presence of some substituent group on the phenol nucleus. An attempt was made to remove any nitroso groups if present by hydrolyzing with 20 per cent hydrochloric acid for 18 hours. The results obtained with these acid hydrolysates (Table II) were very similar to those obtained with the alkaline hydrolysates after reduction except in the case of Deaminized Caseins VII and VIII, where the tyrosine content was lower.

A quantitative study was made of the color production of various substituted phenols with the phenol reagent, using the color developed by tyrosine as a standard. The compounds used were dissolved to give solutions containing the molecular equivalent of 1 mg. of tyrosine per cc. Phenol and the substituted monophenols studied (vanillin, salicylic acid) gave less color than an equivalent amount of tyrosine. The diphenols (catechol, resorcinol, hydroquinone) gave more color than tyrosine but not the theoretical amount expected if both hydroxy groups reacted

[1] It is interesting to note that the values obtained for Deaminized Casein A 64 were similar to, although slightly higher than those previously reported (1). This sample of deaminized casein, prepared and previously analyzed in 1920, had been kept in a brown glass bottle for almost 10 years previous to the second analysis.

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quantitatively. 3,4-dioxyphenylalanine more nearly approached the theoretical value than any other diphenol, giving 174 per cent of the color produced by the molecular equivalent of tyrosine. Phloroglucinol gave more color than tyrosine (121 per cent) but much less than the 300 per cent to be expected if all three hydroxy groups had reacted quantitatively.

These results seemed to indicate that the presence of another group on the benzene ring of phenol influences the reaction with the phenol reagent. In the light of these results, the proof that a part of the tyrosine in deaminized casein contains a second substituent group on the benzene ring would lend weight to the hypothesis that there is no actual destruction of the tyrosine molecule during deamination, but that the decreased chromogenic value with the phenol reagent is merely an expression of a diminished reactivity due to the presence of a substituent group in the tyrosine molecule. With this in mind, an attempt was made to isolate a derivative of tyrosine from the hydrolysate of Deaminized Casein VIII.

On the assumption that the most probable derivative formed in deamination would be either a nitroso or diazo derivative, the hydrolysate was reduced with zinc with the hope that a derivative more basic than tyrosine might be formed, a substance precipitable by some one of the alkaloidal reagents. 5 gm. of Deaminized Casein VIII were hydrolyzed with 20 gm. of barium hydroxide and 100 cc. of water for 48 hours. After the addition of an excess of sulfuric acid and the removal of the barium sulfate by filtration, the filtrate was reduced by adding zinc to the acid solution. The reduction was allowed to proceed for 3 hours at room temperature. After the removal of the sulfuric acid by the addition of a slight excess of barium carbonate and filtration, the filtrate was evaporated on the steam bath to a thick syrup, absolute alcohol added until a slight turbidity developed and an alcoholic solution of picric acid added. After standing overnight in the ice box a yellow needle-like precipitate was deposited. This was recrystallized from the minimum amount of water. This substance, after drying, melted at 256° (uncorrected). A micro analysis (Pregl) for nitrogen showed the presence of 17.18 per cent of nitrogen. The theoretical value for the nitrogen content of the monopicrate of monoamino tyrosine is 17.20 per cent. The

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nitrogen contents of the picrates of the tyrosine itself or of the other amino acids found in the protein molecule do not fall within this range. An unsuccessful attempt to prepare aminotyrosine (13) for comparison was made. It is hoped that a further study may be made of this point.

From these results and in view of the work of Morel and Sisley, we do not consider that any evidence of the destruction of tyrosine in the sense that the molecule is disrupted has been obtained. The decreased color production is probably to be explained on the basis of the formation of a derivative of tyrosine during deamination with a lower chromogenic value with the phenol reagent than tyrosine. The results with the method of Folin and Ciocalteu indicated that a part only of the tyrosine was so affected. This would explain the isolation of tyrosine from the hydrolysates of deaminized casein by Dunn and Lewis (1). It is obvious that colorimetric methods for the determination of tyrosine are unsatisfactory under conditions in which the tyrosine may be modified by the addition of another group to the benzene ring.

The tryptophane contents of deaminized casein and casein were determined by both of the methods suggested by Folin and Ciocalteu. The value obtained for casein (1.30 per cent) was similar to the value obtained by them. The tryptophane content of four different deaminized caseins varied between 1.26 and 1.42 per cent, values closely similar to those obtained for casein, and undoubtedly within the range of experimental error of the method. The failure of deamination to affect the chromogenic value of casein in the tryptophane determination is in accord with the results of Steudel and Schumann (8), who also observed no change in the tryptophane content of deaminized casein as compared with casein.

Basic Amino Acids of Deaminized Casein.

Vickery and his coworkers have recently revised and extended (14, 15) the Kossel method for the separation and quantitative isolation of the basic amino acids of the protein molecule. Their procedure has also been modified by Calvery (16) to make the method available for the analysis of relatively small amounts of protein. Inasmuch as the data concerning the basic amino acids of deaminized proteins are confused and have been obtained by

the older methods, an analysis of Deaminized Casein VIII by the newer methods has been undertaken. After the completion of the work, a similar study of deaminized casein in which methods different from those employed by us were used was reported from the laboratory of Steudel (8).

In general we have followed the technique[2] described by Calvery (16). After the separation of the histidine by precipitation as the

TABLE III.

Basic Amino Acids of Casein.

The results are expressed as percentages of the total N of the protein. Each of the three analyses was made on a separate hydrolysate.

	Analysis 1.	Analysis 2.	Analysis 3.	Average.	Calvery (16).*
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Histidine fraction (precipitated by mercury).					
Total N	3.28	3.05	3.24	3.19	3.43
Hanke-Koessler method (colorimetric)	3.50	3.36	3.36	3.41	3.51
Plimmer-Phillips method (bromination)	3.78	3.97	3.48	3.74	
Isolated as flavianate	2.11	2.38	2.16	2.22	3.01
Arginine fraction.					
Total N	8.64	8.68	8.73	8.68	9.34
N from alkaline hydrolysis	6.55	6.48	6.52	6.52	6.79
Isolated as flavianate	6.10	6.30	6.26	6.22	6.21
Lysine fraction.					
Total N	10.30	9.93	10.70	10.30	10.94
Isolated as picrate	4.72	5.33	5.60	5.22	6.49

* These figures are those obtained by Calvery on a hydrolysate from 50 gm. of casein ((16) p. 639, Table I, and p. 640, Table II, Experiment 5).

mercury salt, we have made use of various methods to determine histidine in the solution obtained after the removal of the mercury from the mercury-histidine precipitate; *i.e.*, colorimetric (Hanke-Koessler), bromination (Plimmer-Phillips), and isolation as flavianate. Similarly in the arginine fraction, we have determined arginine by alkaline hydrolysis and by isolation as the flavianate.

[2] We wish to express our indebtedness to Dr. H. O. Calvery for advice in the use of the procedure for the separation of the basic amino acids.

In the lysine fraction an attempt has been made to isolate and weigh lysine picrate. In all cases, 25 gm. samples of protein have been used for the analyses. The casein from which the deaminized casein was prepared was also analyzed. Fortunately the casein used for analysis by Calvery (16) was the same material so that a comparison of the results obtained by two different investigators on the same sample is possible.

The results obtained with casein are presented in Table III, together with the results obtained by Calvery. All of our own figures are in good agreement with those of Calvery with the possible exception of the values calculated from the weight of histidine isolated as flavianate. The values obtained by Calvery are probably more accurate since a larger sample of casein (50 gm.) was used for analysis and consequently a more complete precipitation of the rather soluble histidine diflavianate should have resulted. The figures obtained by bromination and by the colorimetric method are slightly higher than would have been expected from the total nitrogen of the fraction. It is possible that the higher value obtained by bromination is to be explained by the presence of traces of tyrosine or cystine in the solution, since the former requires 4 and the latter 16 atoms of bromine for complete reaction (17).

In contrast to the results by the Van Slyke procedure (Table I) as already discussed, which indicated the presence of more histidine in deaminized casein than in casein, the isolation method showed a decreased histidine content of casein after deamination (Table IV). The values for casein and deaminized casein were both lower than those reported by Skraup (18), but showed about the same relative decrease. Skraup found 2.6 and 1.18 per cent of histidine in casein and deaminized casein, while the figures presented here, when calculated on the basis of the per cent of histidine in these proteins, represent 1.75 and 0.78 per cent in casein and deaminized casein respectively. This decrease might have been due to the actual destruction of the imidazole ring under the prolonged influence of nitrous acid. Another possible explanation would be the formation of a substituted histidine derivative of decreased basicity which was not precipitated under the same conditions as was histidine. No evidence in support of either of these hypotheses is available.

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The arginine content of casein (Table III), based on the total nitrogen of the fraction, was very similar to the value obtained by the Van Slyke partition method (Table I). The results obtained on hydrolysis of this fraction with sodium hydroxide, however, would indicate that this fraction was not made up of arginine alone. The values obtained by isolation of the arginine as the

TABLE IV.

Basic Amino Acids of Deaminized Casein VIII.

The results are expressed as percentages of the total N of the protein. Each of the analyses was made on a separate hydrolysate.

Analysis No.	1	2	Average.
	per cent	*per cent*	*per cent*
Histidine fraction (precipitated by mercury).			
Total N	1.63	1.29	1.41
Hanke-Koessler method (colorimetric)	1.45	1.28	1.37
Plimmer-Phillips method (bromination)	2.15	2.00	2.07
Isolated as flavianate	1.00	0.93	0.97
Arginine fraction.			
Total N of arginine fraction	1.77	6.50	
N from hydrolysis of arginine fraction	1.77	3.01	
" " " " lysine fraction	4.88	3.55	
Total arginine N by hydrolysis	6.65	6.56	6.61
Isolated as flavianate from arginine fraction	1.84	2.98	
" " " " lysine fraction	1.42	3.26	
Total arginine N from isolation as flavianate	3.26	6.24	
Lysine fraction.			
Total N	12.11	12.41	12.26
N of lysine fraction after correction for arginine N obtained by hydrolysis	7.23	8.86	8.04
Isolated as picrate	0.00	0.00	0.00

flavianate were not as constant as those obtained on alkaline hydrolysis, but represent minimum values, values which are probably close to the true value of the arginine content of casein, since the insolubility of the arginine silver compound and the arginine flavianate make this determination quite accurate. The slightly higher values obtained by alkaline hydrolysis may be due in part to the decomposition of other nitrogenous substances with the liberation of ammonia.

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The arginine fraction of deaminized casein (Table IV) indicated a marked decrease of this amino acid as a result of the action of nitrous acid. Examination of the lysine fraction afforded evidence that this apparent decrease in arginine content was the result of incomplete precipitation of the silver salt of arginine, since on alkaline hydrolysis of the lysine fraction, an amount of nitrogen reacting as arginine nitrogen equivalent to 3 to 4 per cent of the total nitrogen was found. The sum of the values obtained by the hydrolysis of the lysine and arginine fractions with sodium hydroxide was approximately the same as the value found for casein by this method. That the substance present in this lysine fraction which reacted as arginine on alkaline hydrolysis was actually arginine, was proved by the isolation of arginine as the insoluble flavianate as shown in Table IV. In one analysis more than half of the total arginine was isolated as flavianate from the lysine fraction, and the total arginine isolated from the two fractions checked well in this determination with the values obtained for casein. In a second determination, the total arginine as isolated was about half of the value for casein. The appearance of a part of the arginine in the lysine fraction[3] seemed to be of constant occurrence. No difficulty was experienced in effecting a separation of the arginine as the silver salt from the casein hydrolysate, but its precipitation from the hydrolysates of deaminized casein was never complete. The explanation of this is not clear. It is possible that it might have been due to some slight change in the arginine molecule which affected the solubility of its salts or more probably to the interference of some other substances produced in deamination which influenced the solubility of the arginine-silver compound.

The content of lysine in deaminized casein is of greater interest in this study than that of the other amino acids since if the current theory of the position of lysine in the protein molecule is correct, it should have been possible to remove quantitatively the ϵ-amino group of lysine by nitrous acid. The total nitrogen of the lysine fraction of casein (Table IV) agreed well with the values

[3] It would be advantageous to confirm by further studies the presence of arginine in the lysine fraction of deaminized casein by the use of a more specific method of determination; as for example, the arginase method of Hunter and Dauphinee (19).

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found for casein by the Van Slyke procedure (Table I). It should be noted, however, that only slightly more than half of this nitrogen could be isolated as lysine picrate. Leavenworth (20) from the hydrolysate from 1865 gm. of casein was able to isolate 5.77 per cent of lysine as the picrate. This is equivalent to about 7.4 per cent of the total nitrogen of the casein. This may be compared with the figure of 6.49 per cent reported by Calvery (16) and 8.70 and 9.36 per cent by Van Slyke (21). In the latter study, however, the procedure used (Kossel) did not involve actual isolation of the lysine as picrate. Probably the results of Leavenworth are the most accurate because of the large sample of casein hydrolyzed and the repeated crystallization from the mother liquors.

In an attempt to determine what portion of the total nitrogen of the lysine fraction was due to the presence of other nitrogenous substances, an aliquot of the lysine fraction from the casein hydrolysate was subjected to extraction with butyl alcohol for 15 hours, in accordance with the method of Dakin (22). If all the histidine and arginine had been precipitated before the addition of phosphotungstic acid, lysine should have been the only amino acid remaining, which was insoluble in butyl alcohol. About one-third of the nitrogen present was extracted by the alcohol and the residue after the extraction contained nitrogen equivalent to 6.91 per cent of the total nitrogen of the casein used. This checks fairly well with the figure reported for direct isolation by Leavenworth (20) and probably is close to the true value for the lysine nitrogen present in casein.

The total nitrogen content of the lysine fraction of the deaminized casein (Table IV) was much greater than was anticipated, but as previously stated this was due in part to a failure of complete precipitation of the arginine, which resulted in the appearance of arginine in the lysine fraction. After the correction for the arginine content of the lysine fraction was made, the total nitrogen indicated a destruction of about one-fifth of the lysine during deamination. Since no lysine could be isolated as the picrate, this residual nitrogen must be attributed to the occurrence of other amino acids in the lysine fraction. The failure to isolate lysine from the products of hydrolysis of deaminized casein is in accord with the findings of Levites (23).

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In order to demonstrate further the absence of lysine in this fraction an aliquot portion of the lysine fraction of Analysis 1 (Table IV) was extracted for 15 hours with butyl alcohol. The liquid after extraction contained nitrogen equivalent to 4.3 per cent of the total nitrogen of the protein used. Since it has already been shown by alkaline hydrolysis and by direct isolation that this fraction contained about 4.88 per cent of the total nitrogen of the protein molecule in the form of arginine, and since arginine is not extracted by butyl alcohol, this would offer further evidence of the entire destruction of lysine in deamination. About 60 per cent of the nitrogen of the lysine fraction or 6.0 per cent of the total nitrogen of the protein was extracted by the butyl alcohol indicating the presence of monoamino acids in the lysine fraction.

SUMMARY.

1. Casein deaminized by the action of sodium nitrite and acetic acid (1) at various temperatures has been prepared and the deaminized casein analyzed by some of the newer methods of protein analysis.

2. Evidence of the inaccuracy of the Van Slyke method for the determination of characteristic groups of the protein molecule as applied to deaminized casein has been presented.

3. The arginine content of deaminized casein as determined by the Van Slyke procedure was slightly lower than that of casein. This was not substantiated by the results of the Vickery-Leavenworth method of analysis (14), by which practically the same amounts of arginine could be isolated as the flavianate from casein and deaminized casein.

4. The Vickery-Leavenworth method indicated a destruction of more than half of the histidine present in casein in the reactions involved in deamination.

5. The Van Slyke procedure indicated a degree of destruction of lysine varying with the temperature of deamination, while by the Vickery-Leavenworth method no lysine could be isolated from the hydrolysate of casein deaminized at 20°.

6. The tryptophane content of casein, as determined by the colorimetric method of Folin and Ciocalteu was not altered by the reactions of deamination.

7. Tyrosine as determined by the colorimetric methods of Folin

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and his associates, appeared to be partially destroyed in deamination. Evidence has been presented to indicate that this decrease was probably due to the formation of derivatives of tyrosine during deamination, which had less chromogenic value in the color reactions used in the determination of tyrosine than tyrosine itself.

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TISSUE METABOLISM.

I. THE NATURE OF PHLORHIZIN DIABETES.

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The question of the nature of phlorhizin diabetes is still controversial. The interpretation of the extensive chemical, metabolic, respiratory studies of this form of glycosuria, and their significance for human diabetes depend on the exact mechanism involved. Is the glycosuria purely renal in nature, or is it merely one manifestation of a generally distributed disturbance of the mechanism for oxidizing carbohydrate?

Phlorhizin diabetes has been dealt with exhaustively in a recent review by Nash (1) so that only the phases pertinent to the question above will be outlined.

Following von Mering's discovery of this type of glycosuria in 1886, the renal nature of the lesion was apparently demonstrated by Zuntz. The failure to oxidize carbohydrate was laid to the low concentration of carbohydrate in the body, sugar leaking out through the kidneys as soon as it appeared in the blood stream.

The view that there was no intrinsic impairment in the utilization of carbohydrate, was later challenged by Nash and Benedict (2). They administered glucose to phlorhizinized dogs and were able to recover it quantitatively despite the fact that the blood sugar level rose to 3 times the normal for periods of 6 hours or longer. They took this to mean that in spite of the high blood sugar level, no carbohydrate was oxidized, and suggested that phlorhizin acts on the tissues generally to blockade the sugar-burning mechanism of the cell, much as carbon monoxide blocks the oxygen-combining power of hemoglobin. Shortly afterwards, Ringer (3), followed by Gaebler and Murlin (4), observed a rise in the respiratory quotients of

* Working in part under the tenure of a Fellowship in Medicine of the National Research Council.

phlorhizinized animals after the administration of insulin and glucose. These findings were held to support the views of Nash and Benedict as to the extrarenal action of phlorhizin. Ringer maintained that the action was confined to the pancreas, the glucoside preventing the formation of insulin. Nash and Benedict (5), were able, however, to obtain insulin from the pancreas of maximally phlorhizinized dogs.

Further support of the view that phlorhizin interferes with the carbohydrate-oxidizing mechanism of the tissues was offered by Ahlgren (6), who employed the methylene blue technique of Thunberg. He found that minute quantities of phlorhizin prevented the accelerated decolorization of methylene blue that normally followed the addition of glucose and insulin to minced frog muscle. He and also Lundsgaard and Holbøll (7), from their work on minced muscle, came to the conclusion that the action of phlorhizin was to inactivate the muscle complement whose normal function was to convert, in the presence of insulin, the inactive α- and β-glucose into the reactive neo or x form. Further, Gottschalk (8), found that the addition of phlorhizin to liver *in vitro* diminished the formation of acetaldehyde.

The subject was reopened in Lusk's laboratory in 1926 by Wierzuchowski (9) who studied the respiratory metabolism of phlorhizinized dogs immediately after the administration of glucose. He was able to demonstrate rises in the respiratory quotient after glucose alone, as well as the abolition of ketosis and the sparing of protein. In 1927 the first report was made of the experiments which are the subject of this paper (10), as well as the findings of Deuel, Wilson, and Milhorat (11), who obtained high respiratory quotients in phlorhizinized animals which had previously been nephrectomized. They also found high respiratory quotients for 24 hours after the administration of phlorhizin in maximal doses both in oil and in sodium carbonate solutions. These experiments imply that the power of oxidizing carbohydrate is not lost in phlorhizinized animals.

Excised tissue has the advantage of being free of the various disturbing factors such as the low blood sugar, the possibility of storage, and changes in acid-base equilibrium, which may have confused the interpretation of the results obtained by previous investigators. A study was therefore made of the metabolism of the tissues of phlorhizinized animals as to their ability to utilize glucose when removed from the body.

Procedure.

It was first necessary to determine the accuracy of respiratory quotients obtained with the Barcroft-Warburg microrespiration apparatus and the response of normal excised tissue to the addition of glucose. Then phlorhizin glycosuria was induced in rats, and the response to added glucose by the excised tissues of these

animals studied under identical conditions. Since, however, it was possible for the phlorhizinized tissues to have escaped from the influence of the glucoside through its diffusion into the surrounding solutions, phlorhizin in varying concentrations was added to excised normal tissues and to the tissues of completely phlorhizinized animals.

Methods.

White rats (*Mus albinus norvegicus*) were used. They weighed between 200 and 250 gm. and received a ration consisting of two-thirds ground whole wheat, one-third whole milk powder, and containing 2 per cent sodium chloride. From time to time greens were added. During the periods of phlorhizination they were fasted and were kept in metabolism cages of the type devised by Levine and Smith (12), which permitted complete collection of the urine free from feces.

Phlorhizin glycosuria was induced by daily subcutaneous injections of 50 mg. of phlorhizin in olive oil until satisfactory D:N ratios were obtained. The phlorhizin (Merck), was purified after the directions of Deuel and Chambers (13). For most of the experiments the phlorhizin used was one which was later found to give the 2.8 ratio generally in dogs. Another lot of phlorhizin which gave the higher ratio of 3.65 in the dog was then used, with the same level of the D:N and with the same results *in vitro*. This phlorhizin was used with Rats 110 and 111.

In order to establish the fact that the animal as a whole was oxidating no carbohydrate, respiratory quotients were obtained with the intact animal when completely phlorhizinized, preliminary to the study of the tissues *in vitro*. These quotients were obtained in a small respiratory apparatus modified after Haldane (14). Two animals were studied in this way.

The microrespiratory studies were made in apparatus of the form developed by Warburg. A detailed description of the apparatus and the methods of calculating the quotients may be found in the original papers (15) and in the paper by Richardson (16).

The solution in which the tissues were immersed was a Ringer solution isotonic with rat serum and buffered by phosphates in a final concentration of M/150, to pH 7.4. To this, glucose (Kahlbaum) was added to a final concentration of 0.2 per cent when

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desired, and recrystallized phlorhizin (Merck) in varying concentrations.

The tissues used were kidney and testis. The latter consists of long thin tubules which string out easily without trauma. The diameter of the tubules is generally between 0.3 and 0.5 mm. The kidney tissue was prepared by slicing free-hand with a safety razor blade. The thickness of these sections is important for accurate work *in vitro* and can be calculated from the formula developed by Warburg (15). For rat kidney with a maximum oxygen consumption of 25 c.mm. per dry mg. per hour, this critical thickness is 0.367 mm.; for testis, with a maximum oxygen consumption of 10 c.mm. per dry mg. per hour, it is 0.575 mm. The diameters of the tubules measured all fell below this critical thickness. The thickness of the kidney sections was obtained by dividing their area by 5 times their dry weight (a ratio of wet to dry weight of 5:1 being assumed). The dry weight of the tissues was determined after drying to constant weight in an oven at 100°. The areas of most of the sections used were measured. With practice it is possible, with considerable accuracy, to select sections which fall below the critical thickness for kidney, by using the transparency of the section as the criterion. After sectioning, the tissues were placed in the appropriate solutions in the vessels, which were then saturated with pure oxygen for 1 minute, and placed in a constant temperature bath at 37.5°.

Results.

1. Accuracy of Respiratory Quotient Determinations in Vitro and Response of Excised Normal Tissue to Added Glucose.

A series of duplicate respiratory quotients was obtained with excised renal tissue of the rat to determine the accuracy of the methods used in this study.[1] The series was consecutive in that only those observations were discarded in which a technical error could be demonstrated. The data include observations on well fed and fasted rats, and show the variations in the level of the quotients to be expected under these conditions (Table I).

From the duplicate quotients of Table I it may be seen that considerable accuracy can be attained, the mean difference in these

[1] Richardson, H. B., Loebel, R. O., and Shorr, E., *Proc. Soc. Exp. Biol. and Med.*, **24**, 243 (1926) (preliminary report).

fourteen experiments being 0.027, and the error ±0.014. It should be further remarked that every quotient in this series and also the series summarized in Table II falls within physiological limits. The lowest observation was 0.705 which happens to be the theoretical quotient for fat; the highest was 0.949, approaching the level of the exclusive oxidation of carbohydrate. No quotient in excess of unity, or below 0.70, was encountered, such as would indicate the formation of fat from carbohydrate or the reverse.

TABLE I.

Respiratory Quotients of Excised Renal Tissue of Normal Rat; Glucose in Medium.

	Duplicate 1 hr. R.Q.		Difference.
Fed rats.	0.820	0.813	0.007
	0.847	0.823	0.024
	0.760	0.788	0.028
	0.688	0.721	0.033
	0.822	0.802	0.020
	0.845	0.872	0.027
	0.907*	0.774*	
	0.894	0.836	0.058
	0.869	0.819	0.050
	0.796	0.768	0.028
	0.792	0.800	0.008
Fasted rats.	0.861	0.876	0.015
	0.949	0.938	0.011
	0.887	0.843	0.044
Average difference			0.027
" error			±0.014

* Excluded from average because of probable technical error.

These results further imply that excised tissue though deprived of nerve connections and circulation utilizes foodstuffs in the same proportion as does the intact animal.

In the second group of experiments, the ability of excised renal tissue to utilize glucose *in vitro*, was studied. Simultaneous observations were made, with and without glucose in the medium, with tissues of fed and fasted rats. The results are recorded in Table II.

With glucose the quotients are invariably higher and the oxygen consumption is increased. The average increase in quotient with the well fed rats was 0.058, in oxygen consumption, 12 per cent. With the fasted rats the increase in quotient was 0.080 and in oxygen consumption 31 per cent. In both cases the presence of glucose caused an elevation in the respiratory quotient and an increase in oxygen consumption.

TABLE II.

Simultaneous Respiratory Quotients of Excised Renal Tissue of Normal Rat, with and without Glucose in Medium (2 Hour Observations).

	Rat No.	O_2 consumption.			R.Q.		
		No glucose.	0.2 per cent glucose.	Change.	No glucose.	0.2 per cent glucose.	Change.
		c.mm.	*c.mm.*	*per cent*			
Fed rats.	15	38.2	44.8	+17	0.830	0.900	+0.070
	16	34.6	41.9	+21	0.824	0.872	+0.048
	17	33.6	34.6	+3	0.816	0.867	+0.051
	18	37.8	40.7	+8	0.820	0.884	+0.064
Average				+12	0.823	0.881	+0.058
Fasted rats (3 days).	19	30.2	43.8	+45	0.811	0.898	+0.087
	20	34.2	40.6	+17	0.805	0.878	+0.073
Average				+31	0.808	0.888	+0.080
Average of series				+18.5	0.818	0.883	+0.065

2. Response of Excised Renal and Testicular Tissue of Phlorhizinized Rats to Glucose.

Phlorhizin glycosuria was induced in rats in the manner described above. The extremes of the D:N ratios obtained in the eleven animals studied were 2.16 and 4.02. With seven out of the eleven animals the ratios on the day of the experiment fell between 2.51 and 2.90 (Table III).

These figures are as consistent as those obtained with other animals and agree with the lower ratios (17) found in the cat (2.93, 2.80, and 2.93, on the 3rd, 4th, and 5th days of phlorhizination), the goat (2.95, 2.90, and 2.78, on the 2nd, 3rd, and 4th days), and the rabbit (2.89, 2.69 on the 2nd and 3rd days). Anderson

and Anderson (18), using the same dose of phlorhizin in oil but feeding their rats a diet of protein and fat obtained D:N ratios ranging from 2.6 to 3.4. Apparently the higher ratio of 3.65 is found, among the animals so far studied, only in the human subject and the dog. The daily dose of phlorhizin was probably more than sufficient, for smaller doses of 30 and 40 mg. were found adequate and larger doses of 120 mg. failed to raise the D:N.

TABLE III.

D:N Ratios of Phlorhizinized Rats.

Rat No.	Day of phlorhizination.				
	1	2	3	4	5
63	2.71 (a)	3.25 (b)	**2.53** (c,e)		
100	10.40	2.92	2.64	**2.54**	
101	17.10	3.66	2.53	**2.86**	
110	4.74	2.80	**2.63**		
111	3.45	2.16	**2.63**		
55	9.10		3.55	2.84	**2.51**
50	3.23	2.84	2.88	2.78	**2.90**
61	6.92 (a)	2.58 (a)	**2.16**		
65	5.39 (d)		3.14	**3.30**	
49	3.90	2.17	**3.87**		
67	13.50	4.82	**4.02** (f)		

D:N figures on the day used are set in bold face type.

All rats were fasted and given 50 mg. of phlorhizin in olive oil except as noted below:

a = 30 mg.
b = 40 "
c = 120 "
d = 60 "
e = whole animal R.Q. 0.714 in Haldane apparatus.
f = " " " 0.695 " " "

It is about 4 times the customary dose in use for the dog, in terms of the relative weights of the two animals. Several animals died with bloody diarrhea during phlorhizination. They were not autopsied.

Studies of the response to glucose were made with the renal tissue of four, and the testicular tissue of three phlorhizinized rats. The respiratory quotients of two of the latter group were obtained in the modified Haldane apparatus described above, just before

the microrespiratory experiments. These two rats were both found to be oxidizing no carbohydrate, to judge from the respiratory quotients of 0.714 and 0.695 (Table IV).

These experiments, carried out simultaneously on the testicular tissue of the same rat, are characterized, in every case, by a marked

TABLE IV.

Carbohydrate Utilization by Testis of Phlorhizinized Rats (2 Hour Observations).

Rat No.	D:N	Whole rat R.Q.	Metabolism *in vitro*.					
			O_2 consumption.			R.Q.		
			No glucose.	0.2 per cent glucose.	Change.	No glucose.	0.2 per cent glucose.	Change.
			c.mm.	*c.mm.*	*per cent*			
63	2.53	0.714	11.1	18.4	+66	0.755	0.943	+0.188
65	3.30		9.2	16.1	+75	0.683	0.963	+0.280
67	4.04	0.695	8.2	14.0	+70	0.782	0.945	+0.163
Average					+70.2	0.740	0.951	+0.210

TABLE V.

Carbohydrate Utilization by Kidney of Phlorhizinized Rats (2 Hour Observations).

Rat No.	D:N	Metabolism *in vitro*.					
		O_2 consumption.			R.Q.		
		No glucose.	0.2 per cent glucose.	Change.	No glucose.	0.2 per cent glucose.	Change.
		c.mm.	*c.mm.*	*per cent*			
49	3.87	33.4	39.2	+17.5	0.716	0.748	+0.032
50	2.90	38.2	42.3	+11.0	0.667	0.750	+0.083
55	2.51	36.7	43.2	+17.5	0.760	0.798	+0.038
61	2.16	34.3	41.7	+21.5	0.732	0.790	+0.053
Average				+17.0	0.719	0.772	+0.053

increase in the oxygen consumption of the tissues in the presence of glucose and by a corresponding elevation in the respiratory quotient. The average increase in oxygen consumption was 70 per cent, the average elevation of respiratory quotient 0.210, or from 0.740 to 0.951. Thus in the presence of glucose, the phlor-

hizinized testis was oxidizing a mixture of foodstuffs of which carbohydrate formed about 83 per cent. With sections of renal tissue of phlorhizinized rats treated in the same way, an average increase in oxygen consumption of 17 per cent and an average elevation of respiratory quotient of 0.053 were obtained (Table V). These changes are less marked than those found with testis, but are equally definite. They exceed the range of error (± 0.014) of the experiment and duplicate the response of the normal kidney to glucose (Tables II and VIII). The kidney is the only organ definitely known to be affected in the intact animal by phlorhizin (Zuntz). Hence the failure of the drug to cause an inhibition of carbohydrate oxidation in this tissue is of special significance.

These experiments indicate that the renal and testicular tissues of completely phlorhizinized rats are able to oxidize glucose when removed from the body as is evidenced by increased oxygen consumption and rises in the respiratory quotient.

3. Response of Excised Tissues of Normal and Phlorhizinized Rats to Glucose in Presence of Phlorhizin.

A valid objection to these conclusions is the possibility that the drug might have become diluted by diffusion out of the sections of tissue into the surrounding fluid before or during the experiment, the tissues thus escaping from the influence of the glucoside. The following experiments were designed to meet this objection. The principle was to prevent diffusion by supplying an adequate concentration of phlorhizin in the surrounding medium.

In the first group (Table VI), sections of the normal rat kidney were studied in Ringer-phosphate solutions containing 0.2 per cent glucose, some with and some without phlorhizin, in concentrations ranging from 5×10^{-3}, which is a saturated solution at 37.5°, to 10^{-10}. Phlorhizin in concentrations greater than 10^{-4} depresses the respiration of renal tissue. The higher the concentration, the more pronounced is the depression. This narcotic effect suggests that phlorhizin is able to reach the kidney cell *in vitro*. It should then be free to cause whatever other metabolic changes are characteristic of it. The weaker concentrations, 10^{-5} to 10^{-10}, have no effect on the oxygen consumption of the kidney. They fall within the range used by Ahlgren in his experiments with methylene blue in the Thunberg apparatus, and furnish a basis for comparison.

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If phlorhizin interfered with the carbohydrate metabolism of the cell, it should bring about a fall in the respiratory quotient to 0.70, to the level of fat. No such effect was observed. The average respiratory quotient in the absence of phlorhizin was 0.84, in its presence, 0.85. These respiratory quotients indicate a considerable utilization of carbohydrate.

With the second group, the behavior towards glucose of the testicular tissue of completely phlorhizinized rats was studied in media to which phlorhizin was added. The concentration of phlorhizin in the solutions was 10^{-6} to 10^{-5} or approximately that calculated to exist in the tissues of a 200 gm. rat receiving and ex-

TABLE VI.

Effect of Phlorhizin on Respiratory Quotient of Normal Rat Kidney in Vitro; Glucose (0.2 Per Cent) in Medium.

Phlorhizin.	R.Q.		Change in O_2 consumption.	Change in R.Q.
	Without phlorhizin.	With phlorhizin.		
per cent			*per cent*	
5×10^{-3}*		0.84		
5×10^{-3}	0.78	0.82	−42	+0.04
5×10^{-3}	0.92	0.87	−47	−0.05
1.25×10^{-5}	0.88	0.90	−13	+0.02
10^{-6}	0.83	0.82	0	−0.01
10^{-10}	0.80	0.86	+10	+0.06
Average....	0.84	0.85		+0.01

* Saturated.

creting 50 mg. of phlorhizin per 24 hours, on the assumption there was no concentration in any one organ. This is probably somewhat greater than that which actually existed in the tissues, since absorption was intentionally delayed by the use of an oil suspension.

The concentration of phlorhizin used does not affect the oxygen consumption of excised testicular tissue.

In the first experiment of this series (Rat 100), there was no difference in the respiratory quotient in the presence of glucose whether phlorhizin was added or omitted (Table VII). The quotients were nearly unity in both cases. This result serves to sup-

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port the validity of the group of experiments (Tables III and IV) in which phlorhizin was absent from the solutions. The last three were carried out with testicular tissue of completely phlorhizinized rats. The Ringer-phosphate solutions employed all contained phlorhizin in about the concentrations present in the tissues of the animals. Parallel determinations were made with and without glucose. They all confirmed the earlier findings. The oxidation of glucose was indicated by considerable increases in oxygen consumption and corresponding rises in the respiratory

TABLE VII.

Effect of Phlorhizin on Respiratory Quotient of Testis of Phlorhizinized Rat in Vitro; with and without Glucose in Medium.

Rat No.	Solution.	R.Q.			Change in O_2 consumption.	D:N ratio.
		Without phlorhizin.	Phlorhizin 10^{-3}.	Change.		
					per cent	
100	Ringer-glucose-phosphate.	0.97	0.95	−0.02	−9	2.54
		No glucose.	0.2 per cent glucose.	Change.		
101	Ringer-phosphate-phlorhizin 10^{-6}.	0.79	0.93	+0.14	+75.5	2.86
110	Ringer-phosphate-phlorhizin 10^{-5}.	0.72	1.01	+0.29	+89	2.63
111	Same.	0.72	0.91	+0.19	+64	2.63
Average		0.74	0.95	+0.21	+76.2	

quotient. The average increase in oxygen consumption in the presence of glucose was 76 per cent, the average rise of the respiratory quotient was 0.210, or from 0.74 to 0.95, approaching the level of the pure carbohydrate quotient. This corresponds to the range of the respiratory quotient of the testis of the normal rat, with and without glucose, obtained by the same method (Loebel and Hickling (19)), and also with that found for the testis of the intact dog in various nutritional states, by the method of blood gas analysis (Himwich and Nahum (20)).

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DISCUSSION.

These experiments are all consistent in showing that excised renal and testicular tissues of phlorhizinized rats invariably respond to the addition of glucose with an increase in the consumption of oxygen and a concomitant rise in the respiratory quotient. The behavior under these circumstances is identical with that of the same tissue of normal rats. Further, the addition of phlorhizin to excised normal tissue fails to bring about any depression of the respiratory quotient.

These findings are taken to mean that the excised renal and testicular tissues of phlorhizinized rats oxidized glucose with the same readiness as normal tissue. The interpretation of an elevation of the respiratory quotient, in the absence of changes in acid-base equilibrium, or the conversion of carbohydrate to fat, is that it is due to an increase in the relative amount of carbohydrate oxidized. With the increased consumption of oxygen which takes place in these experiments, the rise in respiratory quotient would mean, then, both a relative and an absolute increase in the amount of carbohydrate oxidized by the phlorhizinized tissues. Changes in acid-base equilibrium are avoided by the character of the *in vitro* method. The question of the conversion of one foodstuff into another under these experimental conditions cannot be answered as directly. The respiratory quotient alone would give no evidence of conversion as long as oxidation kept pace with the conversion. Only if the latter were in excess, would the respiratory quotient exceed the normal limits of 0.70 and 1.0. A large number of quotients obtained in this study all fall between 0.70 and 1.0, thus offering no evidence that any such conversion is taking place.

There are three types of evidence supporting the view that the increased oxygen consumption and rise in the respiratory quotient that follow the addition of glucose to the tissues of normal and phlorhizinized rats are due to the oxidation of carbohydrate.

The first pertains to the physiological character of the results obtained with this method of experimentation and to the demonstration of the viability of the tissues under these conditions. In the large series of respiratory quotients assembled in this study, 77 in all, none has fallen outside the range characteristic of the whole animal. This would imply that excised tissues under the con-

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ditions studied, though deprived of nerve and blood supply, utilize the same proportions of foodstuffs as the intact animal. Microscopic examination of the tissues with and without supravital

TABLE VIII.

Effect of Glucose in Vitro on Respiratory Quotient of Excised Tissue of Rat. Comparison of Observed Rise with Theoretical. *

	Rat No.	Change in O_2 consumption.	Change in R.Q.
		per cent	
Kidney of normal rat.	15	+17	+0.070
	16	+21	+0.048
	17	+3	+0.051
	18	+8	+0.064
	19	+45	+0.087
	20	+17	+0.073
Average		+18.5	+0.065
Theoretical			+0.055
Kidney of phlorhizinized rat.	49	+17.5	+0.032
	50	+11.0	+0.083
	55	+17.5	+0.038
	61	+21.5	+0.058
Average		+17.0	+0.053
Theoretical			+0.050
Testis of phlorhizinized rat.	63	+66	+0.188
	65	+75	+0.280
	67	+70	+0.163
	101	+75.5	+0.140
	110	+89	+0.290
	111	+64	+0.190
Average		+73	+0.208
Theoretical			+0.214

* Calculated from the increase in oxygen consumption with glucose, on the assumption that all the rise is at the expense of carbohydrate (*cf.* Zuntz-Schumburg-Lusk table).

staining and the determination of the pH of the cells under our experimental conditions showed these histological criteria of viability to be fulfilled. The pH of the cells was about 6.8 or that generally found to be that of intact living cytoplasm (21). The

staining methods and findings are taken up in detail in the following paper of this series.

There is a second type of evidence supporting the view that the rise in quotient obtained with phlorhizinized tissue in the presence of glucose is actually due to its oxidation. This is furnished by an analysis of our results on the basis of the Zuntz-Schumburg table as modified by Lusk ((17) p. 65) and deals with the possibility that these rises might merely be the expression of a conversion of carbohydrate to fat occurring at ordinary levels of the respiratory quotient. By means of this table, which shows the percentage of the total oxygen consumed by fat and carbohydrate at any level of the respiratory quotient, we can calculate the effect on the quotient of a change in the proportion of these foodstuffs oxidized. Thus, by assuming in our experiments that the increased oxygen consumption in the presence of glucose was entirely due to its oxidation, we could calculate the theoretical rise that should follow. This was done for both normal and phlorhizinized tissue, with the results shown in Table VIII. The striking agreement between the theoretical and the observed rises makes it unnecessary to invoke any other process, such as the conversion of carbohydrate to fat to explain the elevations in quotients. The respiratory quotient for this conversion is 8.0, from the following theoretical formula ((17) p. 396):

$$4C_6H_{12}O_6 + O_2 = \underset{\text{(Palmitic acid.)}}{C_{16}H_{32}O_2} + 8CO_2 + 8H_2O$$

$$\text{R.Q.} = \frac{8CO_2}{O_2} = 8.0$$

From this it follows, that if the elevations in the respiratory quotients were due to the conversion of carbohydrate to fat, they should have taken place with only a small fraction ($\frac{1}{8}$) of the observed increases in oxygen consumption. The increased oxygen consumption shown, for example, in Table IV would have been sufficient to convert enough carbohydrate to fat to raise the quotient to about 3.9. Oxidation of carbohydrate would, on the other hand, account for all the changes quantitatively.

Lastly, if tissues could be found which had lost the capacity to oxidize glucose, the retention of this function by the tissues of phlorhizinized animals would be more significant. Otherwise it

is conceivable that this function is never lost *in vitro*. Our attempts to induce diabetes in the rat by the removal of the pancreas were unsuccessful. By another technique, which entails the removal of the whole intestinal tract and mesentery and tying off the liver, Cori and Cori recently (22) obtained rats which were unable to oxidize glucose without the administration of insulin and were apparently diabetic. We have not as yet studied such tissue *in vitro*. We did, however, produce pancreatic diabetes in the dog. Testicular tissue could not be studied because of difficulties in its preparation for microrespiratory work, but the kidneys and muscle of such animals had the respiratory quotient of fat and failed to show, in the presence of glucose, any increase in oxygen consumption or elevation of the respiratory quotient. These experiments, which indicate an essential difference between the two conditions, are reported in full in the following paper.

The evidence depends on the assumption that phlorhizin is capable of acting outside the body. That some sort of action occurs *in vitro* is shown by the marked depression of respiration caused by the drug. The assumption might, however, be a large one if we were depending entirely on the phlorhizin which is added *in vitro*. In the case of insulin, for instance, the evidence for the action of the extract added to excised tissue is very meager. This is, however, not relevant to the present work. The question is whether the phlorhizin which is given to the intact animal is capable of being transported with the tissue to the experimental vessels. It is not necessary that the extract itself be transferred, but merely some derivative or secondary effect. This question cannot be answered directly, except as a matter of probability. There are, however, analogies in the case of the internal secretions. Removal of the pancreas, as mentioned above, causes a profound depression of the respiratory quotient of excised tissue. Similarly, Foster (23) and Dye and Maughan (24) have shown that the removal of the thyroid causes a depression of the oxygen consumption of excised tissue. The inference is that in the normal tissue the activity of the endogenous hormones is transferred with the tissue to the experimental vessels.

The solutions used in our experiments contained 0.2 per cent glucose. Objection might be raised to the use of such a concentration on the ground that a hypothetical union of phlorhizin

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with the tissue might be disturbed by the mass action of this high concentration of sugar. In phlorhizinized dogs, Nash and Benedict (2) obtained quantitative recoveries when glucose was given in amounts sufficient to produce protracted hyperglycemia. This would imply that if such a union did exist, it was sufficiently stable to prevent carbohydrate oxidation in the face of blood sugar levels as high as the concentrations used in our experiments. That under these conditions carbohydrate oxidation did take place in the excised tissues of phlorhizinized animals is unfavorable to the view that such a combination does exist.

So far we have discussed only the effect of certain procedures on the relative level of the quotient in parallel experiments. In the presence of glucose the elevation was the same whether the tissue was derived from a normal or a phlorhizinized animal. Thus with the kidney, the rise was 0.053 as compared to 0.065 in the normal series. If, however, attention be focussed upon the absolute level of the quotient, it will be seen to be consistently lower with the kidney of the phlorhizinized animals than with the normal group. This does not apply to the testis, which has a quotient of 0.74 without glucose as compared to 0.75 in a normal series (Loebel and Hickling (19)). This tissue when in contact with a solution containing glucose has a quotient near unity whether the animals had received phlorhizin or were untreated (19). With the renal tissue, on the other hand, the quotient of the phlorhizinized animals was 0.719 in the absence of glucose as compared to 0.818 of the normal series. When glucose was present the quotient was 0.772, while that of the normal rats was 0.883. This difference in the level of the quotient is, however, to be expected from our knowledge of normal fasted animals. The rats of the normal series fasted 3 days, whereas those which received phlorhizin fasted from 3 to 5 days and in addition suffered a loss of sugar in the urine. The lower quotients in this latter group are consistent with the more profound tissue starvation which must have resulted from this treatment. The failure to reach the higher levels of the respiratory quotient when glucose was present is also analogous to the response of profoundly starved normal animals to carbohydrate feedings. Such animals do not immediately revert to an exclusively carbohydrate metabolism. The rise in quotient is often extremely small and depends on the degree of undernutrition.

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W. H. Chambers (personal communication) gave 50 gm. of glucose to a normal dog fasted 31 days. The quotient rose from 0.69 to 0.75. Cori and Cori (25) gave to rats fasted 48 hours glucose in quantities sufficient to raise the quotient to unity if it were all oxidized. The quotient rose from 0.71 to 0.84. Chemical analysis of the tissues showed that only 37 per cent was oxidized, the rest being converted to glycogen. In profound states of undernutrition, then, there need be no immediate marked change in the character of the materials oxidized after the administration of glucose.

Our results are in disagreement with those of Ahlgren (6) who employed the methylene blue technique of Thunberg and worked with minced frog muscle *in vacuo*. His index of activity was the rapidity of decolorization of the dye. When glucose, insulin, or phlorhizin (concentration, 10^{-10}) was added alone to the methylene blue-muscle preparation, there was no change in the speed of decolorization. If insulin and glucose were combined, the decolorization was markedly accelerated. The previous addition of phlorhizin prevented this acceleration. If added last, it had no effect. Ahlgren's interpretation of these findings was that phlorhizin inactivated a substance in muscle whose function, in the presence of insulin, was to convert the inactive α- and β-glucose into a reactive neo form. Whichever agent, phlorhizin or insulin, was added first combined with the muscle complement and prevented the other from reacting with it. The reason that phlorhizin alone had no effect on the speed of reaction was that the tissue glucose was already in the reactive form. This theory is largely based on the work of Lundsgaard and Holbøll (7), who reported that insulin in the presence of glucose and fresh muscle was able to form a new-glucose with a different optical activity from α- and β-glucose. They also found that phlorhizin in concentrations greater than 1:50,000 prevented this transformation. Unfortunately, although there have been numerous repetitions of their work, none has confirmed their findings with the possible exception of Bolcato (26). He found, however, that phlorhizin in concentrations as high as 1:10,000 had no effect on the rotation of the muscle-glucose-insulin system.

Apart from the fact that the existence of muscle complement, based on the work of Lundsgaard and Holbøll on neoglucose, lacks

confirmation in other laboratories, there are other objections to this interpretation. It is, at best, still a matter of controversy how far the results obtained with methylene blue and minced muscle *in vacuo* are applicable to the living cell. The variations in behavior of such oxidation-reduction systems when different hydrogen acceptors are used, the failure of cyanides to affect the reactions, are some of the reasons for the position held by Warburg and others that such systems do not faithfully reflect the behavior of the living cell. A more specific objection is the way the tissues were prepared for study. Ahlgren found that the optimum conditions for the decolorization of methylene blue are obtained when the tissues were finely minced for about 5 minutes and even gently ground in a mortar. All the tissues used in his work, including muscle, brain, kidney, and various glands from frog, guinea pig, and rabbit, were treated in this fashion. He found confirmation for this procedure in the experience of Meyerhof (27), that fine mincing of frog muscle also brought about the maximum uptake of oxygen in the Warburg apparatus. But the accelerated respiration of such muscle hash is temporary and is followed by a sharp fall in oxygen consumption, in contrast with the steady respiration for long periods of the intact frog muscle preparation. Furthermore, when Meyerhof and Himwich (28), attempted the same procedure with the diaphragm of the rat, they found that the rate of respiration declined so rapidly at 37.5° that they were forced to abandon this mode of preparation in favor of the intact diaphragm. Our results with relatively untraumatized muscle strips from the dog as compared with muscle hash agree with Meyerhof and Himwich's. The muscle strips respired with great uniformity and with very little falling off in oxygen consumption. Mincing the muscle definitely injured its capacity for respiration in direct proportion to the fineness of the mincing. In muscle minced 5 minutes there was no respiration at all. When we stained this muscle hash with phenol red, it took on the color characteristic of dead or dying tissue and presented other morphological changes found in this state. The relatively untraumatized muscle strips were intact, so far as such methods of examination permit us to judge. So also were the kidney sections and the testicular tissue employed in these experiments.

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Finally, the rate of decolorization of methylene blue can only offer indirect evidence of a disturbance in carbohydrate metabolism. At best, it is a measure of the rate of oxidation, by the removal of hydrogen, of a substrate containing a mixture of substances. Ahlgren's studies give us no information as to the respiratory quotient of the tissues studied or of changes in their carbohydrate content. They therefore lack that type of evidence from which our knowledge of disturbances in carbohydrate metabolism is chiefly derived. The effect of phlorhizin on the insulin-muscle-glucose system, is furthermore, not specific. Ahlgren found that adrenalin and thyroxin also abolished the accelerated decolorization due to insulin. Neither of these substances interferes with the capacity of the living organism to oxidize carbohydrate.

The work of Gottschalk (8), on the influence of phlorhizin on acetaldehyde formation by the liver *in vitro* has also been cited as demonstrating that the effect of phlorhizin on carbohydrate metabolism is generally distributed throughout the tissues. At a concentration of phlorhizin of 2×10^{-4} there was a 30 per cent depression of acetaldehyde formation, at 2.5×10^{-3} a 90 per cent depression. Our experiments indicate a more probable explanation for this reaction than the specific rôle ascribed to phlorhizin. From Table VI it will be seen that phlorhizin in the strong concentrations used by Gottschalk acts as a narcotic to depress oxidation, the depression being greater the higher the concentration. It is more than likely that all the cell processes are slowed down under these circumstances and that the formation of acetaldehyde suffers with the rest. The depression of zymin fermentation by strong concentrations of phlorhizin as reported by Dann and Quastel (29) is work of the same character and subject to the same interpretation.

CONCLUSIONS.

1. The accuracy of respiratory quotients obtained with the Barcroft-Warburg apparatus with excised renal tissue of the rat was found to be ± 0.014. All the quotients fell between 0.705 and 1.00, implying that excised tissues utilized foodstuffs in the same proportions as does the intact animal. Evidence for the conversion of one foodstuff to another was lacking.

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2. Excised renal tissue of the normal rat responded to the presence of glucose by an increase in oxygen consumption and a corresponding rise in the respiratory quotient.

3. Excised renal and testicular tissue of phlorhizinized rats, with and without phlorhizin in the medium, responded to the presence of glucose in the same way as normal tissue by increases in oxygen consumption and corresponding rises in the respiratory quotient.

4. These changes in the presence of glucose were attributed to its oxidation.

5. Excised renal tissue of normal rats in the presence of varying concentrations of phlorhizin suffered no depression of the respiratory quotient. The average of the quotients, 0.850, indicated that carbohydrate was furnishing about 50 per cent of the energy. The narcotic effect of phlorhizin is pointed out.

6. These experiments are against the theory that phlorhizin interferes directly with the oxidation of carbohydrate by the cells in general.

We are indebted to Dr. Stanley R. Benedict for his helpful criticism of this paper.

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TISSUE METABOLISM.

II. THE RESPIRATORY QUOTIENT OF NORMAL AND DIABETIC TISSUE.

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The following work was done to determine the type of foodstuffs utilized by isolated tissues of mammalian organisms.[1] This question is of interest in itself and in relation to the problems of muscular physiology and of diabetes. If the isolated muscle has a respiratory quotient of unity, and can therefore utilize only carbohydrate, then it can make use of fat only after conversion of the latter into carbohydrate elsewhere in the body. If, on the other hand the respiratory quotient is less than 1.0, the muscle must itself be capable of oxidizing fat. Whether in this event the oxidation takes place directly, or through a carbohydrate stage, is not disclosed by the respiratory quotient. Oxidation in isolated tissues is also closely related to the problem of diabetes. Macleod (1928) has revived the theory that the sugar which is excreted in that disease is derived from sources other than carbohydrate, especially fat. One basis for this theory is the belief that the respiratory quotient of isolated tissues is unity, even in diabetic organisms, and that therefore carbohydrate alone is oxidized. Proof that other foods are oxidized would greatly weaken this theory.

This question has been discussed at length in a recent review (Richardson, 1929) in which two sources of error common in the

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[1] A preliminary report was published in *Proc. Soc. Exp. Biol. and Med.*, **25,** 658 (1928).

study of isolated organs have been pointed out. These are first, the effect of a change in the carbon dioxide content of the preparation in the case of the respiratory quotient, and second, the incompleteness of the data of chemical studies. Most of the references in which these factors have been eliminated pertain to the normal rather than the diabetic organism. Meyerhof and Himwich (1924) measured the respiratory quotient of the diaphragm of the fat-fed rat, and found it to be unity. Takane (1926), however, in a later study of the same tissue, concluded that under some circumstances carbohydrate and protein together could not account for the whole of the oxidation. Himwich and Castle (1927) determined the respiratory quotient of muscle *in situ* by analysis of arterial and venous blood. The average was 0.71 ±0.10. Himwich and Rose (1929), using the same method, found in fasted animals an average of 0.78 ± 0.06 after 2 to 15 minutes of exercise. With regard to the organs of diabetic animals the paper of Peserico (1925) may be mentioned. This author studied the respiratory exchange of the excised surviving mammalian heart and stated that the carbon dioxide content of the Ringer solution used for perfusion underwent no noteworthy change in the period of the experiment. He found with depancreatized cats an average respiratory quotient of 0.72 as compared to a range of 0.84 to 0.95 in normal animals. This work contrasts with the results recently reported by Himwich and Nahum (1929) for the brain of the dog. This was studied by means of the gaseous content of arterial and venous blood. The respiratory quotient was found to be exactly unity both in diabetic and normal animals.

The Barcroft manometer, as modified by Warburg (1923) is well adapted to the study of this problem for two reasons: First, the tissues are so treated that most of the cells remain intact, and all of them receive an adequate supply of oxygen; second, the carbon dioxide content both of tissues and fluid is determined at the beginning and end of the observation. The latter factor is included in the calculations, thereby eliminating a prevalent source of error. Another advantage is that two or more parallel quotients can be measured simultaneously. The details of the method and the calculations, together with an illustration of the vessels used, may be found elsewhere (Richardson, 1929). The error as applied to the respiratory quotient of the kidney of the normal rat was

found by Shorr, Loebel, and Richardson (1930) to average ±0.014. The accuracy varies in different tissues. With the kidney of the dog the error in the oxygen consumption averaged ±4 per cent. The maximum variation of the quotient in eight parallel observations was ±0.04 and the average ±0.02. With muscle the greatest variations were ±0.08 in one experiment and ±0.05 in three others. In the remaining twelve it was less than ±0.03, which was the average of the whole group. One experiment on kidney was excluded because of gross variation from the usual result, and one experiment on muscle because of internal evidence of technical defects.

Method.

Dogs were used. Before operation the food consisted of dog biscuit, and after operation, other than pancreatectomy, of a standard ration of cracker meal, meat, lard, and bone ash. Fasting was employed when needed. In the experiments on kidney in 1927 the procedure was to remove the kidney for experiment and immediately afterwards the pancreas. The second observation was performed with the remaining kidney 3 or 4 days later. Thus a majority of the experiments was controlled by measurements made with the same animal prior to removal of the pancreas. In later experiments no special attention was paid to this point.

For anesthesia ether was used, except on two occasions when amytal was given. The advantage of a volatile anesthetic is that it has opportunity to diffuse out of the sections after they are cut, particularly at the time when they are exposed (in fluid) to a current of oxygen for 1 minute. The initial level of the quotient, and, with renal tissue, the increase in oxygen consumption and respiratory quotient when glucose is added to the medium, indicate that in normal dogs the oxidation of carbohydrate persists in spite of the anesthetic. In one experiment in which the rat kidney was taken under ether carbohydrate was found to be utilized to the same degree as in the case of rats which were killed by decapitation.

Pancreatectomy was performed under strict surgical asepsis by Dr. Joshua E. Sweet of the Department of Experimental Surgery at Cornell University Medical College. For completeness of removal chief reliance was placed on the operation itself, since the

postmortem evidence obtained by us in several instances seemed highly inconclusive. As a rule analyses of glucose and nitrogen of the cage urine were also performed. No respiratory determinations of the whole animal were made. After depancreatization the food consisted only of lean meat, 200 to 300 gm. a day, to which 1 gm. of pancreatin was added. The experiment was delayed as long as the condition of the animal would permit; *i.e.*, 3 to 6 days. One experiment, in which insulin was given to facilitate recovery and then withdrawn, gave anomalously high respiratory quotients 0.81 with glucose and 0.87 with lactate. This experiment has not been included in the tables.

The tissue was removed from the animal and received in Ringer's solution or saline in the case of muscle. It was carried to another building for the experiments. There a piece of the kidney was laid on filter paper and cut into sections with the blade of a safety razor. The thickness was judged by the transparency of the section, and the average for each vessel was calculated in twelve of the eighteen experiments, by dividing the wet weight by the area. In every instance but one (see foot-note, Table II) it was found to be within the limiting thickness necessary for the adequate diffusion of oxygen according to the calculations of Warburg (1923). The validity of these calculations as applied to the oxygen consumption of rat kidney has been confirmed in experiments to be reported in a later paper.

The muscle was taken from the neck. Ordinarily the sternomastoid was used, sometimes other muscles of the neck, and on one occasion (February 24) a mixture of strips from different muscles; the tissue was received in physiological saline, staked down with pins on paraffin, and moistened with the same solution. With the aid of fine scissors and forceps the fasciculi were stripped lengthwise, by cutting the intervening fascia. This could be accomplished with little or no cutting of muscle except at the ends. Muscle so treated was found to maintain its initial rate of breathing much better than if subjected to mincing, which is the procedure in common use. For example, in six vessels containing strips the oxygen consumption decreased on the average only 12 per cent in the 2nd hour of observation as compared to the first, whereas simultaneously in two vessels containing hash of the same muscle the decline was 49 per cent. By sufficiently fine mincing the

respiration can be reduced to a tenth of the usual value. The average thickness of the strips was not determined, but was not enough to interfere with accuracy if one may judge by the fact that in parallel determinations the variation between vessels averaged ±5 per cent. It is to be noted that with muscle the permissible thickness is 2.5 times as great as with the kidney, because the oxygen consumption is very much less (2.5 compared to 15 c.mm.), and the limit is inversely proportional to the square root of oxygen consumption.

After preparation the tissue was transferred to Warburg vessels of the type illustrated elsewhere (Richardson, 1929). These contained fluid which was buffered to pH 7.4 by the use of phosphate mixtures in a final concentration of M/150. The usual Ringer's solution was used for kidney, but was found to have a deleterious effect on the respiration of muscle. In a preliminary experiment, in which the muscle was minced, the consumption of oxygen in Ringer's solution was less than half of that observed in saline. Presumably the difference is due to calcium, which is known to have an injurious action on muscle. Whether this is true also of strips was not determined. In all subsequent experiments with muscle the potassium and calcium were omitted from the Ringer's solution. Except for these omissions the two solutions were made up in exactly the same way. The final concentration of sodium chloride was 7.78 gm. per liter for Ringer's solution and 8.1 gm. per liter for buffered saline.

The air in the apparatus was replaced by oxygen and measurements made at 37.5° by the use of manometers, according to the usual technique. The time elapsed from removal of the kidney to setting the vessel in the bath was from 23 to 90 minutes, averaging about 50 minutes. Similar figures for muscle were 55 to 85 minutes, averaging about 60 minutes. After about 20 minutes in the bath the actual measurements were begun. The chemicals were obtained from Kahlbaum. The Ringer's solution was made up according to the formula used by Warburg (1923) and had a depression of the freezing point of Δ 0.56. The sodium lactate was of the racemic type and was neutralized by the addition of 0.1 N sodium hydroxide.

The utilization of foodstuffs *in vitro* was studied in two ways: (1) the rate of respiration and the level of the respiratory quotient

without foodstuff, and (2) the influence on these of adding glucose or lactate to the surrounding fluid. The material to be studied was weighed out and added to the solution in which the tissue was to be immersed. In each experiment two or more quotients were obtained simultaneously with the same organ.

Results.

Results obtained *in vitro* are of more value if they are applicable to conditions within the body. There are several indications that the tissues were in a relatively normal condition. Extraneous factors might be expected to produce variable results, in contrast to the rather narrow margin of error actually found in parallel experiments. With renal tissue the variation in the oxygen consumption and respiratory quotient averaged ± 4 per cent and ± 0.02 respectively. With muscle the figures were ± 5.4 per cent and ± 0.03. The constancy exhibited in different experiments under like conditions may be seen in Tables II to V. Survival of tissue is also suggested when respiration continues at the initial rate. The decline in the rate of respiration of normal tissue in the 2nd hour of observation as compared to the 1st was only 8 per cent for kidney and 17 per cent for muscle. The respiratory quotients gave no evidence of abnormal metabolism. With very rare exceptions they were within the limits usual for the intact animal; *i.e.*, from 0.70 to 1.00. Analogies were found *in vitro* to the response of the whole organism to altered dietary conditions. Sections of kidney showed the influence of glucose or sodium lactate by a rise in the oxygen consumption and respiratory quotient. Previous fasting had the opposite effect both on kidney and on muscle. The state of the tissues was also judged by direct observations, made with the help of Mr. Herbert Pollack of Cornell University Medical College as follows:

The sections, both of kidney and of muscle appeared histologically normal after an experiment; no granulation of the cytoplasm had taken place. The muscle strips had an intact cellular structure, whereas the minced muscle displayed disintegration in about 50 per cent of the cells. Furthermore the muscle strips twitched when pricked with a needle. Neither the renal tissue nor the strips of muscle stained either with phenol red or brom-cresol purple, in contrast to the minced muscle in which about half of the cells

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TABLE I.

Oxygen Consumption of Isolated Tissue of Dog per Mg. of Dried Tissue per Hour. Excised Tissue Compared to Organs in Situ.

Author.	O_2 consumption.			Remarks.
	Maximum.	Minimum.	Average.	
Kidney *in situ*, method of Barcroft and Brodie.				
	c.mm.	*c.mm.*	*c.mm.*	
Barcroft and Brodie (1905-06).	4.8	22.5	8.6	Four experiments shown in Table III Period 1, without diuretics.
Bainbridge and Evans (1914).	12.0	14.0		Used heart-lung-kidney preparation. Two experiments. 21.0 c.mm. after sodium sulfate.
Bornstein and Gremels (1928).	20	30	25.3	Heart-lung-kidney preparation, perfused.
Tamura, Watanabe, *et al.* (1926).	16			One observation. Slightly less after caffeine.
Hayman and Schmidt (1927).	8	22	15.9	Four determinations.
Excised renal tissue, method of Warburg.				
Grafe, Reinwein, and Singer (1925).			14.1	No. of experiments not specified.
Present work.	12.8	14.8	13.5	Animal fed, glucose in Ringer's solution.
	10.1	15.4	12.5	Animal fed, no glucose in Ringer's solution.
	16.4	19.7	18.1	Animal fed, lactate in Ringer's solution.
	11.2	11.8	11.5	Animal fasted, glucose in Ringer's solution.
	9.3	12.1	11.0	Animal fasted, no glucose in Ringer's solution.
Muscle *in situ*, method of Barcroft and Brodie.				
Jannsen and Jost (1925).	0.95	2.90	2.23	Four experiments. On previous day spinal cord section under hedonal.
Himwich and Castle (1927).	1.12	2.80	1.78	Decerebrate dogs; fasted 3 days or longer. Five experiments.

TABLE I—*Concluded.*

Author.	O_2 consumption.			Remarks.
	Maximum.	Minimum.	Average.	
Excised muscle in strips, method of Warburg.				
	c.mm.	*c.mm.*	*c.mm.*	
Present work.	1.90	2.92	2.43	Animals fed. 0.2 per cent glucose in medium.
	1.66	2.27	2.00	Animals fasted. With and without glucose in the medium.

were stained yellow with phenol red, indicating a permeability not possessed by normal cells (Evans and Schulemann, 1914). To fix the pH of the tissues they were dropped into boiling water (Fletcher and Hopkins, 1906–07). On staining, the protoplasm became blue to brom-cresol purple and was therefore above pH 6.6, and yellow to phenol, hence below pH 7.0. This range corresponds to the values obtained by Schmidtmann (1925) using the micro manipulator of Péterfi for the study of animal tissue. In small mammals and lower animals the pH of the protoplasm of renal tubules was 6.9 to 7.4, and of heart and voluntary muscle 6.7 to 6.8. Using a similar method Chambers, Pollack, and Hiller (1927) obtained a value of 6.9 $\pm$ 0.1 pH for various tissues of frog and necturus.

Finally, the respiration of excised sections or strips was compared with the respiration of the same organs *in situ* as disclosed in the literature (Table I). Data are taken from papers in which the oxygen consumption is determined *in situ* by analysis of arterial and venous blood in conjunction with the rate of blood flow; *i.e.*, the method of Barcroft and Brodie (1905–06). The results are compared with those obtained by the method of Warburg. All figures are reduced to c.mm. per mg. of dry weight per hour, on the assumption that the latter is one-fifth of the volume. This figure was found in ten determinations to be correct as an average for the muscle of the dog.

Inspection of Table I shows that the values obtained with the Warburg apparatus are at the same general level as those quoted for tissues *in situ*, both with kidney and muscle. Grafe, Reinwein, and Singer's figure for kidney sections is the same as our own.

TABLE II.

Oxygen Consumption of Excised Renal Tissue of Dog per Mg. of Dry Weight per Hour.

Date.	Foodstuff in medium. None.	Glucose 0.2 per cent.	Lactate 0.01 M.	D:N*	Remarks.
Normal animals.					
1927	*c.mm.*	*c.mm.*	*c.mm.*		
Apr. 11	10.1†	12.8†			
May 7	12.2	14.8			
" 9	12.1	13.4			
" 16	15.4		18.2		
" 23		13.4	19.7		
June 1		13.3	16.4		
" 6		13.4			
Average.....	12.5	13.5	18.1		
Normal, fasted.					
1929					
Feb. 28	12.1	11.6†			Fasted 11 days.
Mar. 11	9.3†	11.2†			" 10½ "
" 26	11.6†	11.8†			" 11 "
Average.....	11.0	11.5			
Depancreatized.					
1927					
Apr. 14	17.1†	18.2†		2.7	Depancreatized 3 days previous. 24 hr. urine.
May 13	17.1	17.2	19.1	1.8	Depancreatized 4 days previous. Urine 10 a.m.–1.40 p.m.
" 19	17.4		21.3‡	3.2	Depancreatized 3 days previous. Urine 10 a.m.–1.30 p.m.
" 27		17.3	21.0	3.1	Depancreatized 4 days previous. 24 hr. urine.
1928					
Mar. 27	17.1	15.2	17.2		Depancreatized 4 days previous.
" 28	19.4	21.9	24.0		Same dog as in previous experiment.
Average.....	17.6	18.0	20.5		

To obtain the approximate oxygen consumption per mg. of *moist* weight multiply by 0.2. Observations recorded on the same horizontal line are simultaneous.

* Dextrose-nitrogen ratio of cage urine on day of experiment.

† Average of two simultaneous observations. All others are single determinations.

‡ The tissue in this vessel was about 20 per cent thicker than the limiting thickness.

TABLE III.

Respiratory Quotient of Excised Kidney of Dog.

Date.	Foodstuff in medium. None.	Glucose 0.2 per cent.	Lactate 0.01 M.	D:N*	Remarks.
Normal animals.					
1927					
Apr. 11	0.76	0.86			
May 7	0.79	0.87			
" 9	0.86	0.90			
" 16	0.85		0.93		
" 23	0.89		0.97		
June 1		0.87	0.90		
" 6		0.86†			
Average.....	0.81	0.87	0.93		
Normal, fasted.					
1929					
Feb. 28	0.73†	0.77†			Fasted 11 days.
Mar. 11	0.78†	0.83†			" 10½ "
" 26	0.78†	0.84†			" 11 "
Average.....	0.76	0.81			
Depancreatized.					
1927					
Apr. 14	0.67	0.68		2.7	Depancreatized 3 days previous.
May 13	0.71	0.68		1.8	" 4 " "
" 19	0.71		0.76	3.2	" 3 " "
" 27		0.70	0.73	3.1	" 4 " "
1928					
Mar. 27	0.77†	0.77†	0.78†		" 4 " "
" 28	0.70†	0.76†	0.72†		Same dog as in previous experiment.
Average.....	0.71	0.72	0.75		

Observations recorded on the same horizontal line are simultaneous.

* Dextrose-nitrogen ratio of cage urine for 24 hour period ending on the day of experiment.

† Average of two simultaneous observations. All others are single determinations.

The constancy of the values for excised tissues is in striking contrast with the variations reported *in situ*. This suggests that the advantage is, if anything, in favor of the Warburg apparatus.

Further results may be grouped according to the tissue used, kidney or muscle. The oxygen consumption of the kidney is shown in Table II in which the figures denote c.mm. of oxygen per mg. of tissue per hour, the weight being determined subsequent to the observation, after the tissue was dried to a constant value. In this and the subsequent tables the respiratory data on the same horizontal line were obtained simultaneously. It will be seen that the respiration of excised renal tissue was remarkably constant under like conditions. In the organ taken from the normal fed dog there was an increase of 8 per cent when glucose was added to the Ringer's solution in which the tissue was immersed. In fasted animals the increase was less marked. The effect of sodium lactate was striking and amounted to 44 per cent. In the tissue taken from the depancreatized dog no increase as a result of glucose could be demonstrated. Sodium lactate caused a rise of 16 per cent, compared to 44 per cent in the normal. If the results are compared in another way, it will be seen that there was a definite decrease in the respiration as a result of fasting. A pronounced increase was observed in the depancreatized dogs.

In Table III are shown the respiratory quotients which were obtained in the experiments of Table II. With the fed animal, without foodstuff in the solution, the range was from 0.76 to 0.89, and the average 0.81. When glucose was added to the Ringer's solution, the average quotient was 0.87, and when lactate was added, 0.93. Thus the influence of carbohydrate was definitely seen in the respiratory quotient. In the fasted animals the initial level was lower but the same degree of rise was noted as a result of the glucose; namely, from 0.76 to 0.81. This occurred in spite of the fact that the oxygen consumption was not appreciably affected. With the depancreatized dog the respiratory quotients (Table III) averaged 0.71 without glucose in the Ringer's solution, and 0.72 with glucose. With four of the five animals the quotients were between 0.68 and 0.71 in the absence of lactate. Use of the latter caused a rise in two experiments, and none in two others. The average with lactate was 0.75. If the experiments in which no lactate was used are excluded, the averages were 0.73 and 0.74

TABLE IV.

Oxygen Consumption of Excised Muscle of Dog per Mg. of Dry Weight per Hour.

Date.	Foodstuff in medium. None.	Glucose 0.2 per cent.	Lactate 0.01 M.	D:N*	Remarks.
Normal animals.					
1928	*c.mm.*	*c.mm.*	*c.mm.*		
Feb. 6		2.92			
" 7		2.82			
" 10		2.07			
" 9		1.90			Amytal anesthesia.
Average.....		2.43			
Normal, fasted.					
Feb. 23	1.83	1.82†	1.82		Fasted 5 days.
" 16	1.80†	2.27			" 5 " 40 gm. dog biscuit on morning of experiment.
Mar. 1	2.18	2.16			Fasted 6 days. Same animal.
" 2	2.18	1.66			" 7 " " "
Average.....	2.00	1.98			
Depancreatized.					
Feb. 14		2.41		1.7	Depancreatized 4 days previous. 24 hr. urine.
" 15		2.56		2.6	Same animal. 24 hr. urine.
" 18–20				4.0	Depancreatized 3 days previous. 48 hr. urine.
" 20		2.39			
" 21	2.93		2.81	2.2	Same dog. 24 hr. urine.
" 24	2.53	2.33	2.54†	1.6	Depancreatized 6 days previous. 24 hr. urine.
" 22		2.68		6.2	Depancreatized 4 days previous. Muscle removed under amytal anesthesia. Urine, 30 cc. from bladder.
Average.....		2.51			

Observations recorded on the same horizontal line are simultaneous.

* Dextrose-nitrogen ratio of cage urine.

† Single determination. All others are averages of two or three simultaneous observations.

TABLE V.

Respiratory Quotient of Excised Muscle of Dog.

Date.	Foodstuff in medium. None.	Glucose 0.2 per cent.	Lactate 0.01 M.	D:N*	Remarks.
Normal animals.					
1923					
Feb. 6		0.81			
" 7		0.90			
" 9		0.91			Amytal anesthesia.
Average.....		0.87			
Normal, fasted.					
Feb. 23	0.82	0.84†			Fasted 5 days.
" 16	0.74†	0.83			" 5 " 40 gm. dog biscuit on morning of operation.
Mar. 1	0.70†	0.77			Fasted 6 days. Same animal.
" 2	0.80	0.76			" 7 " " "
Average	0.77	0.80			
Depancreatized.					
Feb. 14		0.72		1.7	Depancreatized 4 days previous. 24 hr. specimen of urine.
" 15		0.73		2.6	Same animal. 24 hr. urine.
" 18–20				4.0	Depancreatized 3 days previous. 48 hr. urine.
" 20		0.71			
" 21			0.76	2.2	Same dog. 24 hr. urine.
" 24	0.71	0.74		1.6	Depancreatized 6 days previous. 24 hr. urine.
May 22		0.74		6.2	Depancreatized 4 days previous. Muscle removed under amytal anesthesia. Urine, 30 cc. from bladder.
Average.....		0.73			

Observations recorded on the same horizontal lines are simultaneous.

* Dextrose-nitrogen ratio of cage urine.

† Single determination. All others are averages of two or three simultaneous observations.

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without lactate, and 0.75 with it. This difference is scarcely large enough to be significant. If, however, the results are viewed in conjunction with the increase in oxygen consumption due to lactate, an increase in the oxidation of carbohydrate becomes possible.

With the excised muscle of the normal dog the oxygen consumption (Q_{O_2}) was very constant, as may be seen in Table IV, although less so than was noted with the kidney. It was not influenced by the presence of foodstuff (glucose or sodium lactate) in the saline solution, whether the animals were fasted or depancreatized. Deprivation of food, however, was attended by a fall in the average oxygen consumption from 2.43 to 1.98 c.mm. Removal of the pancreas followed by a meat ration resulted in the consumption of oxygen at the same rate as in the fed animal.

The respiratory quotients of the same experiments are shown in Table V. With tissue from a normal fed animal these varied from 0.81 to 0.91 in three experiments. The fact that they did not reach unity is significant, since there was more than enough glucose in the medium to permit this. The influence of the nutritional state was evidenced in the fasted animal by a somewhat lower level, 0.80 against 0.87, but no consistent increase as the result of glucose was noted. With the depancreatized dog the quotients were low, 0.71 to 0.74, averaging 0.73, in spite of the presence of glucose in the solution. This is a little above the level which might be expected from the combustion of pure fat. In an experiment without foodstuff the figure was 0.71 and in another with lactate it was 0.76. The latter was not attended by a rise in the oxygen consumption. Thus after removal of the pancreas there was very little oxidation of carbohydrate. Evidence of oxidation was confined to two points, first, that the level of the quotient was somewhat higher than might be expected of diabetic tissue, and second, that with the kidney there was a definite though moderate increase in the consumption of oxygen in the presence of lactate.

DISCUSSION.

In the foregoing work much of the difficulty in the study of excised muscle was obviated by the use of strips rather than minced muscle. That both this preparation and the sections of kidney, when studied in the Warburg apparatus, were in a relatively normal condition, has been indicated in the preceding section.

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With tissue from normal animals the respiratory quotient ranged from 0.76 to 0.97. Quotients of the same order were obtained by Evans (1913–14) with the heart-lung preparation, by Peserico (1925) with the excised mammalian heart, by Himwich and Castle (1927) with resting muscle *in situ*, by Himwich and Rose (1929) with exercising muscle, and by Himwich and Nahum (1929) with testis. In four experiments with the diaphragm of the rat Takane (1926) obtained figures ranging from 0.66 to 0.90, and concluded from other data that the oxidation of carbohydrate did not necessarily account for the entire utilization of oxygen. It is evident that fat enters the metabolism of isolated tissue of the organs mentioned on equal terms with carbohydrate. The depression of the respiratory quotient and oxygen consumption by fasting (Tables II to IV) and the increase, in the case of the kidney, when glucose or lactate is added to the solution, indicate that the proportions in which foodstuffs were oxidized varied in response to altered dietary conditions. Analogous results were obtained by Evans (1913–14) with the heart-lung preparation. The above evidence is against the contention that isolated tissue necessarily respires with a respiratory quotient of unity, and therefore against the theory that muscle is dependent exclusively on carbohydrate for its immediate fuel. It seems definitely to exclude the idea that fat is necessarily converted into carbohydrate in one organ for oxidation in another. This conversion might take place without effect on the respiratory quotient, but only within the organ concerned.

The brain of the normal dog, in contact with its blood supply, appears to differ from the other organs mentioned. The respiratory quotients measured by Himwich and Nahum (1929) were always unity. That these results may have been influenced by the presence of food in the blood is suggested by the work of Loebel (1925) on the gray matter of the rat. This tissue was found to have a respiratory quotient of 0.86 in Ringer's solution alone, 0.92 with the addition of lactate, and 0.99 with fructose.

One effect of the removal of the pancreas was to cause an increase in the oxygen consumption of the excised kidney. This might have been due, in the first four experiments, to the previous removal of one kidney, but this explanation did not apply to the remaining two experiments. Other possibilities are stimulation

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of the metabolism in general by the diabetes, or stimulation of the kidney by the presence of abnormal excretory products.

With excised kidney or muscle taken from a depancreatized animal the respiratory quotient averaged 0.71 to 0.75, or so low as to preclude the direct oxidation of any considerable amount of carbohydrate. Furthermore, the rise in breathing and respiratory quotient observed in the kidney of normal animals as a result of adding glucose to the solution was absent in the diabetic organ. The only definite evidence of a response of diabetic tissue to the presence of carbohydrate in the solution was the moderate increase in oxygen consumption observed with lactate.

As with normal tissue, the data are against the belief that preformed glucose is indispensable as a fuel for oxidation in isolated organs. If glucose is oxidized to any considerable extent in the tissues, it must be manufactured locally. In order to produce the narrow range of quotients actually observed the supply must be closely adjusted to the demand. Little margin would be left to account for the waste glucose which is characteristic of diabetes.

The present findings are in agreement with the work of Peserico (1925) who found in the excised surviving heart of the depancreatized cat respiratory quotients varying from 0.62 to 0.80, with an average of 0.72. They are, however, in contrast to those of Himwich and Nahum (1929), who found that the respiratory quotient of the isolated brain *in situ* was 1.0 in depancreatized dogs as well as in normals. This might be used as an argument in favor of the neoproduction of glucose. Other explanations, apart from the use of different methods, are that (1) some tissues retain insulin longer than others, (2) that some tissues have the power of oxidizing lactic acid or carbohydrate without insulin, and (3) that an unknown substance is oxidized with a respiratory quotient of unity. As to the first suggestion, Nothmann (1925) has found that the brain, among other organs, loses its normal content of extractable insulin after pancreatectomy.

Whether the quotients observed in the present work indicate the oxidation of a little carbohydrate or none at all, is not clear. In man the theoretical level of the respiratory quotient, below which oxidation of carbohydrate is excluded, has been calculated to be 0.66 (Richardson, 1929). It is essentially that of the fatty acid of fat when oxidized to β-hydroxybutyric acid, since theoretically,

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when no sugar is oxidized, not only the carbohydrate, but also the glycerol of the fat, as well as nearly all of the protein molecule, is withdrawn from oxidation. The last follows from the consideration that the unoxidized products of protein metabolism, *i.e.* glucose, keto acid, and nitrogenous waste, amount to 93 per cent of the total. These figures are based on the estimate that human fat has a respiratory quotient of 0.707. In the dog the respiratory quotient of fat is somewhat higher (Krzywanek, 1925), depending on whether beef fat is oxidized at 0.711, or dog fat at 0.723. In the depancreatized dog, moreover, the dextrose-nitrogen ratio is nearer 2.8 than 3.65 and the waste of glucose is correspondingly less, hence it is possible that a certain amount of protein is oxidized. If the respiratory quotient of protein be taken at 0.80, the waste of sugar corresponding to a D:N of 2.8 would reduce it to 0.695.

On the other hand, it is unnecessary to assume that in complete diabetes any protein is oxidized. Even in the normal animal this may depend on some specialized organ such as the liver, and therefore be absent from excised tissue. For the conflicting literature on formation of ammonia, reference may be made to Bollman, Mann, and Magath (1926), Nash and Benedict (1921), Bliss (1926), Benedict and Nash (1926), Bliss (1928), Gottlieb (1928), Meyerhof, Lohmann, and Meier (1925), Emden and Wassemeyer (1928), Parnas (1928, 1929).

In the present state of knowledge, then, it is uncertain to what degree the quotients herein reported exceed the theoretical level below which no oxidation of carbohydrate is possible. A more definite indication of this oxidation is the increase in the oxygen consumption of the diabetic kidney in the presence of lactate, combined with a slight rise in the respiratory quotient. A more marked increase in the latter would be required to prove the oxidation of carbohydrate rather than a stimulus of respiration in general. For instance, it is possible that the increase in respiration might indicate the use of non-carbohydrate substances as energy for the conversion of lactic acid into glucose. Assuming that this explanation does not hold, it may be possible that lactate has a special sensitivity to oxidation. Other explanations have already been suggested; namely, that the tissues retain a certain amount of insulin, temporarily at least, that they are capable to a certain extent of oxidizing lactic acid or other carbohydrate in the

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entire absence of insulin, or that some other substance is oxidized with a respiratory quotient above that of fat. The insulin content of organs has been studied by subjecting them to the chemical procedures used in the preparation of the extract. Best, Smith, and Scott (1924) extracted the organs of normal and diabetic dogs, and found in both a substance which reduced the blood sugar of test animals. The presence of insulin in diabetic tissues was not confirmed, however, either by Ashby (1923) or Nothmann (1925). In a very carefully controlled investigation the latter could detect no insulin in the tissues of depancreatized as contrasted with normal dogs, except for a normal amount in the liver. Several organs were examined. The brain was extracted 5, 15, and 5 days after pancreatectomy, the kidney in three instances 5 days after operation, and muscle in ten experiments 2 to 15 days after. There is therefore little evidence to date that insulin persists in the organs of the depancreatized dogs, except in the liver.

Whether from persistence of insulin or other cause, the effect of pancreatectomy is not immediate. This is indicated by the work of Verzár and von Fejér (1913) who studied the effect of the injection of sugar in the depancreatized dog. There was a rise in the respiratory quotient up to the 4th day with glucose, and up to the 9th day with levulose (Verzár, 1914).

In the foregoing data the evidence of the direct oxidation of carbohydrate by excised tissue of depancreatized animals is comparatively slight. The data as a whole constitute strong evidence in favor of the theory that diabetes in the depancreatized animal is due to a defect in the oxidation of carbohydrate.

CONCLUSIONS.

1. The rate of oxidation in excised renal or muscular tissue of the dog varies within narrow limits, and is about the same as is found in the intact organ *in situ*.

2. The respiratory quotients of normal tissues are intermediate between 1.0 and 0.70, and imply the participation of fat in the oxidation.

3. The average respiratory quotient of renal or muscular tissue taken from depancreatized dogs was 0.70 to 0.75, indicating either no oxidation of carbohydrate, or at most a small amount.

4. These findings conflict with the theory that carbohydrate is essential as the immediate fuel for muscle.

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5. These findings also conflict with that theory of diabetes which holds that the waste glucose originates from fat.

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THE OCCURRENCE OF *D*-MANNOSE IN SEAWEED AND THE SEPARATION OF *L*-FUCOSE AND *D*-MANNOSE.*

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(Received for publication, February 7, 1930.)

The wide-spread occurrence of mannans and fucosans in nature makes it not improbable that some of these vegetable gums may in fact consist in part at least of fuco-mannans. In general the isolation and identification of two or more sugars from a mixture is often fraught with experimental difficulties, particularly if the material is available only in small amounts. Any simple procedure applicable to small quantities which facilitates the separate identification of sugars is certain, therefore, to find a use.

The author had occasion to prepare comparatively large quantities of *L*-fucose by the excellent method of Clark[1] from seaweed (*Fucus vesiculosus*, L.) gathered on the southern shores of the Baltic Sea. As will appear in the sequel, the method, however, is unfortunate when appreciable amounts of mannose are present because the fucose phenylhydrazone will be contaminated with the sparingly soluble mannose phenylhydrazone and the product regenerated from this is a mixture. Fortunately enough the first crops of sugar appear to be entirely free of mannose but the crystals isolated from the mother liquor have been shown to contain mannose in not inappreciable amounts. Furthermore, the final mother liquor which crystallized only with difficulty contained approximately 10 per cent mannose.

This concentration of mannose in the mother liquors makes possible its isolation and identification by two different methods which in their practical application the author believes to be new.

* The capital *D* and *L* denote configurational relationships without consideration of the actual signs of rotation which are indicated customarily by the lower case *d* and *l*.

[1] Clark, E. P., *J. Biol. Chem.*, **54**, 68 (1922).

EXPERIMENTAL.

The directions of Clark[1] for the isolation of fucose were closely adhered to. The first mother liquor on concentration yielded a second crop of colorless crystals in which the presence of *D*-mannose was shown as follows: To 16.4 gm. of the sugar dissolved in 100 cc. of water were added 5 cc. of liquid hydrogen cyanide and a drop of aqueous ammonia. The mixture was allowed to remain on ice for 3 days, when a test portion failed to reduce Fehling's solution appreciably. Some amide had crystallized from the solution which had turned slightly brown. The mixture was hydrolyzed with a slight excess of barium hydroxide, decolorized with norit, filtered, and allowed to cool. In a short time a copious yield of a sparingly soluble barium salt was obtained which was dissolved in hot water and freed quantitatively of barium by means of dilute sulfuric acid. The colorless solution was concentrated to about 40 cc. on the water bath. On cooling and standing for several days 1.5 gm. of colorless needle-shaped crystals separated which after one recrystallization began to sinter at 170°[2] and melted to a clear liquid at 172° with some effervescence due to the loss of water. This behavior is identical with that of δ-*D*-mannoheptoic lactone[3] which was synthesized for comparison by the method of Fischer and Passmore[4] from an authentic specimen of mannose. No difference in behavior was observed on mixing the two products. The identity was further confirmed by conversion to the lactone which alone or admixed with an authentic specimen[4] melted at 150–151°.

Isolation of Mannose Phenylhydrazone.—The sugars in the final mother liquor referred to above were converted into phenylhydrazones.[1] The pale yellow product was recrystallized once from 60 per cent alcohol, filtered, and washed. To 15 gm. of the dried material were added 75 cc. of cold pyridine. In a few minutes the fucose phenylhydrazone had completely dissolved,

[2] Melting points are corrected.

[3] The prefix δ is used to designate that the configuration of the added asymmetric carbon atom is such that the hydroxyl group is on the right if the conventional aldehyde structure of the sugar is written vertically with the aldehyde group at the top. On this basis the epimer is referred to as λ-*D*-mannoheptoic lactone.

[4] Fischer, E., and Passmore, F., *Ber. chem. Ges.*, **23**, 2226 (1890).

leaving a residue of colorless crystals of mannose phenylhydrazone. The latter is surprizingly insoluble in pyridine even when warmed. The crystals were filtered off and washed with 30 cc. of pyridine in small portions and finally with much dry ether. The product (1.5 gm.) was recrystallized from 150 cc. of 60 per cent alcohol and then melted either alone or admixed with an authentic specimen of *D*-mannose phenylhydrazone at 194°. Examination under the petrographic microscope failed to disclose any differences.

The fucose phenylhydrazone is readily obtained from the pyridine solution by cautious precipitation with dry ether, 400 cc. being used in the experiment here described. Recrystallization from hot water with the aid of a little norit yielded pure fucose phenylhydrazone which melted at 175–176°. Admixture with an authentic specimen did not depress the melting point.

SUMMARY.

1. Mannose has been identified as a constituent of the hydrolytic product from *Fucus vesiculosus*, L.

2. The greater portion of the fucose was removed by crystallization and in a less pure although crystalline product the presence of *D*-mannose was shown by the isolation of a mannoheptoic acid and lactone from the hydrogen cyanide addition product.

3. Mannose had concentrated in the final mother liquor and the isolation of its phenylhydrazone was made possible by the remarkable insolubility of the latter in pyridine.

In conclusion the author expresses his thanks to Professor R. J. Anderson who noticed the heterogeneity of the phenylhydrazone while examining it for another purpose, and to Dr. E. J. Roberts who kindly made the crystallographic measurements.

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NOTE ON THE ACTION OF THE COLON BACILLUS UPON THE NON-GLUCOSE REDUCING SUBSTANCES IN HUMAN BLOOD.*

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(Received for publication, January 16, 1930.)

In a previous report (2) the action of the colon bacillus upon the reducing compounds of human blood was described. It was shown that after incubation of neutralized Folin-Wu filtrate with this organism, which attacks all the simple reducing carbohydrates, there were still present definite compounds which reduced the copper solution used in the Folin-Wu blood sugar method. These compounds were not produced by the growth of the colon bacillus, nor were they introduced during the analysis. Their concentration was about 3 times as great in whole blood as in plasma.

In the present communication results of the application of a similar technique to filtrates which are free from glucose are described. This filtrate was prepared in the manner described by Benedict (3). Large amounts of yeast were added to blood or plasma, incubation carried on for about 10 minutes, and the yeast and protein removed together by the action of tungstic acid. The solution was then pasteurized by heating at 80° and brought approximately to neutrality by the addition of alkali. In preference to dilute sodium hydroxide, which was tested and found moderately satisfactory for this purpose, a sterilized phosphate buffer solution containing NaH_2PO_4, 6.4 per cent, and Na_2HPO_4, 17.9 per cent, was used. Usually 1 to 2 drops of this solution for each cc. of filtrate were required. Filtrate so prepared was then inoculated with an organism which had the morphological and cultural characteristics of *Bacillus coli communis*, and placed in an

* This work formed part of a paper read before the meeting of the American Society of Biological Chemistry at Ann Arbor, April, 1928 (1).

incubator at 38°. Experiments showed that part of the non-glucose reducing compounds present are unstable in neutral or alkaline solution, and it was therefore necessary to run a control through all the steps of the procedure. The reducing action of the control and of the inoculated solutions was determined after 18 to 24 hours—longer incubation up to about 72 hours did not affect the results—by a slight modification of the Folin-Wu blood sugar method (2). About 100 specimens of blood and plasma were studied as described, and in all of them the specimen treated with colon bacilli showed a lower concentration of reducing compounds than did the control. The amount destroyed by the organism varied somewhat in different blood samples: The average, expressed in terms of its glucose equivalent, was approximately 3 mg. per 100 cc. of blood. In contradistinction to most of the other non-glucose reducing compounds of blood, these substances seemed to be present in approximately the same concentration in plasma and corpuscles. In whole blood they formed only about a fifth of all the non-glucose reducing compounds present, but in most specimens of plasma approximately half of the reducing action was due to compounds destroyed by the colon bacillus.

A few experiments were carried out to determine what these compounds might be. Uric acid, creatine, and creatinine are the most important known reducing compounds in blood which are distributed equally between cells and plasma. These were added to blood and it was found that they did not affect the determination. The simple reducing sugars, glucose and levulose, were added to the whole blood, but, in common with saccharose, they were wholly removed by the treatment with yeast. When lactose and galactose were added to blood, however, the results were different. These sugars were not affected by yeast at all, and were recovered quantitatively in the filtrate from the tungstic acid precipitation. When these filtrates were inoculated with the colon bacillus, however, these sugars, as well as maltose, which was partially fermented by the yeast, were completely destroyed. It is evident that if these compounds, or sugars of a similar nature, had been present in the original blood samples they would have given the results described above: there would have been more reduction from glucose-free filtrates and from incubated control solutions than there was in solutions which had been inoculated with the colon bacillus.

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Although the results can be explained by the assumption that blood contains simple reducing sugars which are not fermented by yeast, they do not justify one in concluding that such compounds are actually present, for the complex mixture obtained by precipitating blood with tungstic acid may include reducing substances of an entirely different nature which are destroyed by the colon bacillus.

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THE METABOLISM OF THE PHOSPHOLIPIDS.

I. THE INFLUENCE OF DIET ON THE AMOUNT AND COMPOSITION OF THE PHOSPHOLIPID FATTY ACIDS IN VARIOUS TISSUES OF THE CAT.*

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INTRODUCTION.

From the mass of information concerning the phospholipids of animal tissues several facts are outstanding: one is the marked constancy in the percentage amount present under widely different nutritional conditions (Mayer and Schaeffer, 1913); another is the apparent relationship between the functional activity of a tissue and its phospholipid content (Bloor, 1926, 1927, 1928); and still another is the high degree of unsaturation of their constituent fatty acids as compared with depot fat. The general conclusion has been that the phospholipids are undoubtedly essential constituents of protoplasm and fulfil a very important function in the vital reactions of the cell, but surprizingly little is known concerning the precise nature of this function and the purpose of the high degree of unsaturation.

As compared with the amount of work that has been done on the influence of ingested fat on the character of the fat deposited in the animal stores (see Anderson and Mendel, 1928; Eckstein, 1929), very little attention has been paid to the possible effect of food fat on the composition of the tissue phospholipids. Such investigations as have been made have been concerned, for the

* The substance of this paper was presented at The Thirteenth International Physiological Congress held at Boston in August, 1929 (Sinclair and Bloor (1929)).

most part, with the effect of food fat either on the degree of unsaturation of the phospholipids in the liver (Joannovics and Pick, 1910; Shioji, 1924) or on the composition of the lecithin in the eggs of laying hens (McCollum, Halpin, and Drescher, 1912–13). The findings of these various workers agree in showing that the degree of unsaturation of the phospholipids is influenced to a very appreciable extent by the type of fat in the diet.

Recently Terroine and Belin (1927) have come to quite the opposite conclusion. On the basis of a comprehensive study of the fatty acids of the *élément constant* (which are, according to these authors, the phospholipid fatty acids) of various animals and animal tissues, they concluded that the *composition* as well as the *amount* of the phospholipid in a tissue is a fixed characteristic of that particular tissue and wholly independent both of the species of the animal and of the nature of the fat in the diet.

In a recent paper (1929) the author presented evidence to show that the fatty acids of the food fat are transformed into phospholipid in the intestinal mucosa and in the liver within a few hours after its ingestion, presumably as a stage in the resynthesis into neutral fat. At the same time attention was called to the fact that the degree of unsaturation of the phospholipid fatty acids in the intestinal muscle is unmistakably higher in cats fed on beef kidney than in those fed on meat scraps. Since the total fatty acids of beef kidney are, on the average, more unsaturated than those of beef muscle (Bloor, 1927, 1928) the obvious interpretation is that food fat in due time does exert an influence on the composition of the tissue phospholipids.

In view of the contradiction between our own observations and those of Terroine and Belin, and, moreover, because of its great importance for the proper understanding of the function of the phospholipids, it was deemed advisable to obtain a definite answer to the question of whether or not the composition of the phospholipids is influenced by the character of the food fat.

In the present paper data are presented which show beyond any doubt that the degree of unsaturation of the phospholipid fatty acids of the liver, heart, kidneys, muscle, intestinal mucosa, and probably the brain of the cat is dependent to a very considerable extent upon the type of diet fed.

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Procedure.

Since it had been found that the phospholipids of the intestinal and skeletal muscles of the cat are not affected by ingested fat during the period of absorption (Sinclair, 1929), it was concluded that the apparent effect of diet on the composition of the muscle phospholipids was probably a gradual process. Consequently all cats used in the present investigation were maintained on one or the other of the two diets for at least 2 weeks and usually longer before they were killed.

Diets.—In choosing diets the first requirement of course was that there should be a considerable difference in the degree of unsaturation of their total fatty acids. However, other factors had to be considered. Owing to the length of time over which the feeding had to be continued, it was necessary that the diets should remain reasonably constant in composition from day to day. Furthermore since cats are very particular about their food, the diets had to be of such a nature that they would be readily consumed. All of these requirements were nicely filled by beef kidney and lean beef muscle. From the work of Bloor (1927, 1928) it has been calculated that the total fatty acids in beef kidney have, on the average, an iodine number (I.N.) of 98, while those of beef muscle (in this case the round was used) have an average I.N. of 82. The difference between these average I.N. is not as great, nor is the composition of these tissues as uniform, as might be wished for, but these faults were compensated by the absence of any difficulty in getting the cats to eat their food regularly.

Preparation of Tissues for Extraction.—The cats were anesthetized with illuminating gas and then bled to death.

In order to obviate as much as possible postmortem changes, every effort was made to have the hashed tissues immersed in alcohol in the shortest time possible.[1] Since four tissues from each cat were used and only two of them could be extracted at one time, it was necessary to preserve the other two immersed in cold alcohol in the dark for 6 to 8 hours before extraction. In order that preservation of the tissue in this way should not influence the comparative value of the results, the order in which the tissues were analyzed was alternated.

[1] The author is indebted to Mrs. Jane Fagan for valuable assistance in preparing the tissues for extraction.

Extraction and Separation of Lipids. The procedure followed in extracting and separating the lipids was essentially that used by Bloor (1926). Briefly, it involves extraction of the hashed tissue with hot alcohol in a continuous extractor, evaporation of the alcohol under reduced pressure, extraction of the residue with moist ether, and separation into an acetone-soluble (fat and cholesterol) and an acetone-insoluble fraction (phospholipids) by the addition of acetone. A small amount of $MgCl_2$ in alcoholic solution was used to aid complete precipitation of the acetone-insoluble substances. The acetone-insoluble fraction was freed from fat by scrubbing once with acetone and then reprecipitating with acetone from the ether solution. The total acetone-insoluble fraction—except in the case of brain, where only the ether-soluble phospholipids were taken—was saponified for 3 to 4 hours with NaOH in 50 per cent alcoholic solution. After acidification, the fatty acids were extracted with petroleum ether, dried on the steam bath in a current of CO_2, and weighed. The Hanus method was used for the determination of the iodine number.

It is essential to emphasize that—except for brain—the term "phospholipid fatty acids" is used to indicate the fatty acids obtained by the saponification of all the acetone-insoluble lipids.

DISCUSSION.

The data on the amount and I.N. of the phospholipid fatty acids in the various tissues of the cat are condensed in Table I. In every case the value given represents the average of seven cats, except for intestinal mucosa, intestinal muscle, and heart with the kidney diet, in which cases five, two, and six cats, respectively, were used. Since the purpose of the investivation was to compare the phospholipid fatty acids in the tissues of cats fed on a kidney diet with those of cats fed on beef muscle with respect to their amount and degree of unsaturation, it was deemed advisable to subject the data to statistical treatment. To this end, the standard deviation of the individual values from the mean has been calculated by the use of the formula $\sigma = \sqrt{\frac{\Sigma^2}{n}}$. In addition, a mathematical expression of the significance of the differences between the average values for the two diets has been obtained by

TABLE I.

Influence of Diet on Phospholipid Fatty Acids of the Cat.

Tissue.	Weight of phospholipid fatty acids per 100 gm. moist tissue.			Weight of phospholipid fatty acids per 100 gm. dry extracted tissue.			Iodine No. of phospholipid fatty acids.		
	Diet.		Probability difference is not significant.	Diet.		Probability difference is not significant.	Diet.		Probability difference is not significant.
	Beef kidney.	Beef muscle.		Beef kidney.	Beef muscle.		Beef kidney.	Beef muscle.	
	gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*			*per cent*
Brain	4.82±0.256	5.00±0.218	58.9	43.0 ±2.93	44.2 ±2.09	74.1	105±2.4	100±1.8	9.5
Liver	2.47±0.133	1.94±0.217	3.8	10.23±0.898	7.98±1.210	13.7	145±2.5	124±8.0	1.2
Kidney	2.11±0.136	2.04±0.211	78.1	13.71±0.895	12.86±1.062	54.2	112±1.9	95±3.8	0.006
Mucosa	1.28±0.069	1.29±0.055	91.2	8.42±0.348	8.62±0.372	69.7	114±1.3	97±2.0	0.2×10^{-8}
Heart	1.27±0.095	1.25±0.088	88.1	8.69±1.239	7.78±0.603	50.9	140±3.6	121±3.2	0.009
Striated muscle	0.70±0.102	0.61±0.039	40.8	3.38±0.603	3.21±0.244	79.5	131±6.3	108±2.7	0.08
Smooth "	0.63	0.64±0.097		3.44	3.47±0.644		109±4.3*	92±4.0	0.37

* The average of seven experiments, five of which were taken from earlier work (Sinclair, 1929).

using the formula $\frac{\bar{x}}{\sigma}$ in which $\bar{x}$ is the difference between the means and $\sigma = \sqrt{(\sigma_1)^2 + (\sigma_2)^2}$, σ_1 and σ_2 being the standard deviations from each of the means (Dunn, 1929, pp. 310, 341).

Influence of Diet on Amount of Phospholipid.—The data in Table I show that the phospholipid content of all the tissues with the exception of the liver is the same in the cats fed beef kidney as in those fed on beef muscle. This is no more than is to be expected in the light of the observations of Mayer and Schaeffer (1913) that the diet has no influence on the content of ether-soluble phosphorus in tissues.

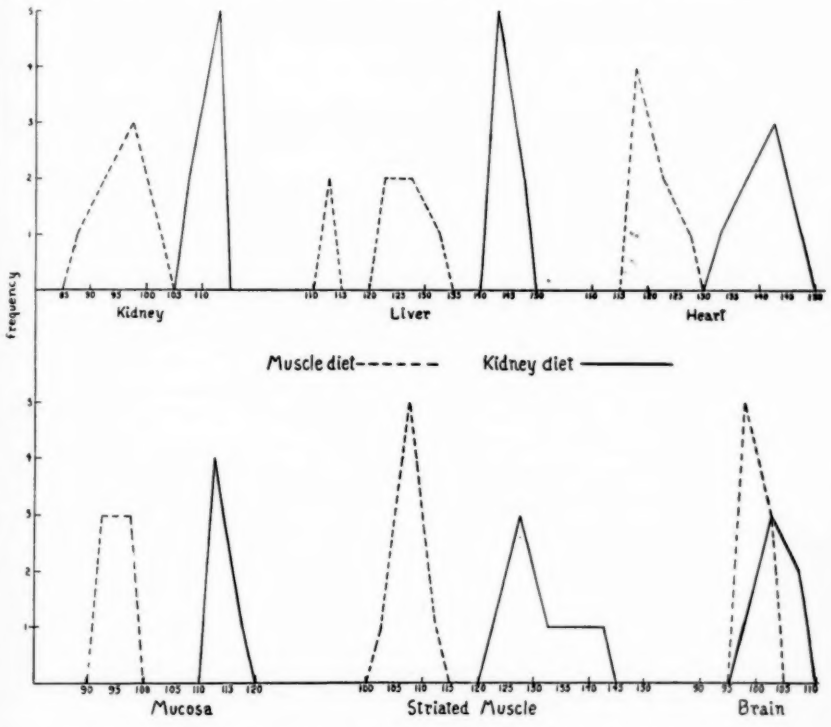
On the other hand, it is quite apparent that the livers of cats fed on beef kidney contain more (27 per cent) phospholipid fatty acids than those of cats fed on beef muscle. It seems probable that the explanation of this difference lies in the fact that the phospholipid content of beef kidney is about 7 times that of the round muscle, as shown by the work of Bloor (1927, 1928).

Influence of Diet on Composition of Phospholipids.—The data in the last three columns of Table I prove beyond a doubt that the degree of unsaturation of the phospholipids of the various tissues of the cat is influenced by the character of the fat in the diet. Only in the case of the brain is there any uncertainty as to the significance of the difference between the average I.N. These facts are brought out very clearly by the frequency curves given in Fig 1.

Attention has already been called to the fact that in the case of the brain only the ether-soluble substances (lecithin and cephalin) in the acetone-insoluble fraction were saponified. This was done because in the first three experiments, in which the regular procedure was followed, the very low values of 79, 84, and 83 for the I.N. were obtained. Removal of the substances insoluble in ether resulted in higher I.N., but even then the brain did not yield clear cut results as did the other tissues. This fact is hardly to be wondered at, since it is not improbable that the phospholipids of the brain fulfil a different—or an additional—function from that of the phospholipids in the parenchymatous and muscular tissues.

The fact that the fat in the diet governs to a very considerable extent the composition of the tissue phospholipids might be in-

terpreted in two ways: first, that it is evidence in favor of the belief that the phospholipids are intermediary in the metabolism of fat; second, that it shows that phospholipids, being essential



VARIATION IN IODINE NUMBERS OF PHOSPHOLIPID FATTY ACIDS OF THE CAT

FIG. 1. Curves showing the frequency of distribution of the iodine numbers of the phospholipid fatty acids in various tissues of cats fed on beef kidney (continuous lines) and on beef muscle (dotted lines).

constituents of the protoplasm, are subject to continual wear and tear and, just as ingested amino acids are used in the repair of tissue protein, so the fatty acids of food fat are used in the repair

of tissue phospholipids. Until more is known concerning the rate at which the composition of the phospholipids changes under the stimulus of a change in the character of the diet, it is considered inadvisable to attempt to decide as to which of the above interpretations is correct.

SUMMARY.

A study has been made of the question of whether or not the composition of the phospholipids in animal tissues is influenced by the character of the fat in the diet.

It has been found that the degree of unsaturation of the phospholipid fatty acids in the liver, heart, kidneys, smooth and skeletal muscle, intestinal mucosa, and probably the brain is consistently higher when cats are fed on an exclusive diet of beef kidney than when they are fed on hashed beef muscle.

This fact may indicate either that phospholipid is intermediary in the metabolism of fat or that the tissue phosholipids undergo continual wear and tear and replacement at the expense of food fat.

The amount of phospholipid fatty acids in all tissues except the liver is the same for both diets; in the liver there is a greater content of phospholipid when beef kidney is fed than when the diet consists of beef muscle.

The author takes pleasure in acknowledging his indebtedness to Professor Bloor for counsel and helpful criticism throughout this investigation.

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ON THE NATURE AND RÔLE OF THE FATTY ACIDS ESSENTIAL IN NUTRITION.*

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In our introductory paper on this subject (1) it was shown that when rats were reared on a fat-free diet a deficiency disease developed which had not been previously described. This disease is rather specific since the scaly condition of the skin develops while growth is continuing at an approximately normal rate. Later the tail often becomes necrotic and the kidneys degenerate, allowing the passage of blood into the urine. The animals always die at an early age unless fed a curative dose of fat. The fatty acid fraction is the only part of the fat effective in curing or preventing the disease. When a curative dose is fed, the extremely emaciated animals begin to grow and store depot fat.

In the present communication the authors will discuss the following topics: further observation on the effects of the disease; effect of protein intake on the severity of the disease; effect of fat exclusion on water exchange; effect of fat exclusion on ovulation and reproduction; nature of the essential fatty acids.

Further Observations on Effects of the Disease.

In our earlier paper the necrosis of the tail was greatly emphasized as a condition due to the fat deficiency. During the past year much of the work has involved the testing of substances by the cure method already described. For this work animals which have been on the fat-free diet only 4 to 6 months after weaning can be used since they have already reached a growth plateau or are declining in weight. But at this age many animals have not developed severe lesions of the tail. And when they have done

* This work was supported largely by a grant from the Medical Research Fund of the University of Minnesota.

so these lesions are often so severe that they require many weeks to heal. The most sensitive test of the disease is the scaliness of the feet, especially the hind feet. This always appears within a few weeks after the young animal is put on the fat-free diet and will disappear completely in 3 or 4 weeks after a good oil is fed. Another reliable indication of the condition of the animal is dandruff on the back. The feet and back are always carefully described in the data for cure work.

The condition of the kidneys should be emphasized much more than was done in the earlier paper. In fact, most of the animals on the fat-free diet which have been killed during the past year have shown grossly abnormal kidneys. Even the breaking down of the kidneys to such a degree that blood appears in the urine is very common and this observation may be used as a measure of the severity of the disease. It now seems probable that in most cases the immediate cause of death of the animal is kidney degeneration. This kidney degeneration must not be confused with that due to lack of vitamin A. It has been shown that increased doses of Fraction AD do not improve the animal, while the addition of vitamin A-free fatty acids[1] or oils does. That Fraction AD is readily absorbed in the absence of fat was shown by the very early decline and death of animals from which Fraction AD was withheld ((1) p. 364). The animals receiving no fat grow to twice the size of those receiving fat but no Fraction AD and live several times as long. Furthermore, the animals receiving the low fat diet show no signs of xerophthalmia throughout life.

We may, therefore, consider growth, scaliness of the skin, and condition of the kidneys as measures of the effect of various fats in the diet, all other known factors being excluded or constant. Other measures will be discussed in following sections.[2]

Effect of Protein Intake on Severity of the Disease.

While convenient quantitative measures of the severity of the disease due to low fat diet were being sought, the question of ideal

[1] Fraction AD designates the non-saponifiable matter of cod liver oil prepared and fed as described in our earlier paper. Fraction E designates the concentrated vitamin E prepared as described below.

[2] The histopathology of the kidneys will soon be reported by Professor C. M. Jackson.

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diet for producing the disease arose. There were only the two constituents of the diet to be varied, casein and sucrose, the salts being kept constant except for the ash of the casein. Since the disease involves the kidneys, it seemed likely that merely increasing the per cent of protein in the diet would increase the severity

TABLE I.

Effect of High Protein Diet and Low Protein Diet on Rats.

Male rats were used throughout.

Group No. and diet.	Rat No.	Maximum weight.	Condition of skin (scale and dandruff).	Condition of urine.
		gm.		
Group 58. Diet 550 + 0.65 gm. yeast + Fraction AD + Fraction E.	2913	214	Medium.	Bloody.
	BH29536	210	Much.	No blood.
	W29531	178	Great deal.	Much "
	GH29533	160	" "	Bloody.
	2908	242	" "	Great deal of blood.
	2920	205	Extreme.	No blood.
Average		201		
Group 59. Diet 550 B + 0.65 gm. yeast + Fraction AD + Fraction E.	BH29532	196	Medium.	No blood.
	W29530	185	"	" "
	2909	245	Great deal.	" "
	2915	211	Extreme.	Much "
	W29540	208	Great deal.	No "
	W29534	187	" "	" "
Average		205		
Controls. 4 rats on Diet 550. 4 on Diet 550 B + 0.65 gm. yeast + Fraction AD + Fraction E + 10 drops lard.		273	Normal.	Normal.

of the disease and hasten the decline of the animal. There has been lack of agreement generally as to whether high protein diets caused the lesions of the kidneys known as nephritis and nephrosis, but recent workers (2–4) have agreed that exceedingly high protein diets do not produce kidney lesions.

To furnish further facts concerning the breakdown of the kidney in the present work as well as to check the above work the following experiment was performed. Twelve young male rats were divided into two groups (Nos. 58 and 59). Group 58 received Diet 550[3] + 0.65 gm. of yeast + Fraction AD + Fraction E. Group 59 received Diet 550 B + 0.65 gm. of yeast + Fraction AD + Fraction E. The two groups were therefore receiving identical diets except that one diet contains twice as much casein as the other. The results are summarized in Table I. The maximum weights may be considered equivalent. The condition of the skin is so bad that no general difference between the two groups could be detected. But when the numbers of cases with bloody urine are compared, there is evidence that the high protein diet has increased the injury to the kidneys. Four of the six in Group 58 showed bloody urine before autopsy (8 months old), while only one of the six in Group 59, low protein, showed bloody urine. These groups are small and the evidence cannot be considered as final. But if these results can be repeated (or made even more marked by using greater differences in the protein contents of the diets), then it must be concluded that high protein intake does aid in the breakdown of the kidney when the kidney is already weakened by the absence of essential fatty acids. From the histological studies of Professor Jackson we will know whether even the kidneys of those animals in which there was no blood in the urine differed in degree of degeneration. No kidney degeneration was detected in the controls (Table I).

Effect of Fat Exclusion on Water Exchange.

Early in the course of this work it was noticed that those animals on the fat-free diets consumed more water than their controls receiving some fat. Water consumption was soon put on a quantitative basis and recorded with the same regularity as food consumption. Both values have been known throughout this work. All experimental animals are kept in the individual round cages described in the earlier paper. The food cup is of a type which

[3] For these diets see Burr and Burr (1). Diet 550 contains casein 24 per cent, sucrose 72.1 per cent, salts (*cf.* (1)) 3.9 per cent, nutritive ratio 1:3. Diet 550 B contains casein 12 per cent, sucrose 84.1 per cent, salts 3.9 per cent, nutritive ratio 1:7.

permits almost no spilling, and these records can be kept with considerable accuracy. The water is kept in 250 cc. bottles filled to the 250 cc. mark on the neck. They are stoppered with No. 6 rubber stoppers carrying a bent glass tip made of 10 mm. tubing. These tips are blown with especially high lips about the opening which is on top of the nearly horizontal arm. Almost no detectable spilling takes place. The bottles do not vary more than 5 cc. in volume. The chief cause of spilling from these bottles when first used was found to be the expansion and contraction of the air in the bottle when the room temperature fluctuated greatly. But for nearly 2 years now the room has been kept at a constant temperature with a maximum daily fluctuation of $\pm 1°$. The amount of spilling has been found to be extremely little in those cages which have been tested by placing them over funnels. The difference in urine excretions when the animal is watered outside the cage and when watered by the bottle is not appreciable. Any spilling of water would have increased the apparent urine volume.

It should be pointed out here that water consumption differs greatly among individuals of the same size and on the same diet so that individual cages must be used to get any true picture of the variations. The same statement applies to food consumption.

Table II gives a comparison of actual volumes of water consumed, expressed in cc. per day for individuals with and without fat in the diet. Each water consumption period is from 7 to 12 days in length, depending upon the rate the bottles are emptied. Daily measurements are not made. The bottles are filled, left until nearly empty, the residual water measured, and the total consumption divided by the length of the period in days. Only a few of the groups and a few periods for these groups can be given here because of lack of space. The data given here are typical and will illustrate the variation among members of the same group and the variation of the same individual from week to week. It should be noted that these values represent the water consumed from the bottles only. The daily yeast dose is fed with 4.5 cc. of water. Therefore, for total water consumed by each animal add 4.5 cc. to the amount given in Table II.

Although there is considerable variation among the members of the same group, it is evident from Table II that this variation is

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TABLE II.

Typical Water Consumption Records for Animals with and without Fat in Diet.

Female rats were used except in the cases noted.

Group No. and diet.	Amount of fat in diet.	Rat No.	Weight of rats Nov. 17.	Water consumption per day for period.					
				Oct. 22–30.	Oct. 30–Nov. 7.	Nov. 7–14.	Nov. 14–23.	Nov. 23–Dec. 1.	Dec. 1–10.
			gm.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Group 3. Diet 550 B + Y* + Fraction AD.	None.	27139	144	23.8	24.4	23.6	25.5	26.9	28.3
		27278	171	21.2	20.0	20.7	21.7	24.4	24.4
		27123	162	15.0	16.3	17.1	20.0	22.5	26.1
Group 4. Same + lard.	10 drops lard daily.	27132	214	10.0	11.3	10.0	9.4	10.0	10.6
		27261	156	16.2	13.1	11.4	15.6	14.4	13.9
		27122	180	10.6	11.3	10.0	9.4	9.4	10.0
Group 19. Diet 560 B + Y + Fraction AD.	20% lard in diet.	27202	191	9.4	9.4	10.0	11.1	11.9	11.7
		27186	201	10.0	10.0	9.3	8.3	10.6	9.4
		27190	185	11.9	11.9	12.9	12.2	12.5	12.2
Group 56. Same as for Group 3.	None.	28276	123	20.0	20.0	19.3	18.9	19.4	21.1
		28282	131	20.0	18.1	19.3	18.3	21.3	21.7
		28281	112	20.0	19.4	21.4	20.6	20.6	Died.
		28275♂	166	18.8	18.1	18.6	18.9	18.8	19.4
Group 57. Same as for Group 19.	20% lard in diet.	28277	160	8.8	8.8	7.1	9.4	8.8	8.9
		28280	184	10.6	11.9	10.7	9.4	11.3	12.2
		28279	190	8.1	8.3	8.3	7.8	9.4	9.4
		28278♂	202	9.4	10.7	10.7	10.6	11.3	11.1
Average for no fat groups			144	19.8	19.5	20.0	20.6	22.0	23.5
" " 10 drops lard group			183	12.3	11.9	10.5	11.5	11.3	11.5
" " 20 per cent lard groups			188	9.7	10.1	9.9	9.8	10.8	10.7

Average daily water consumption.

Fat-free	20.9 cc.
10 drops lard	11.5 "
20 per cent lard	10.2 "

* Y designates the daily dose of 0.65 gm. of ether-extracted yeast, as described in the earlier paper.

small as compared with the average difference between groups with and without fat. It is also evident that 10 drops of lard (2 per cent of total diet) cut down water consumption just as effectively as 20 per cent lard in the diet. Only a few cases have been given here but these results apply uniformly to all rats reared during the past 2 years. On examination of all records it is found that among those animals receiving 10 drops of an oil there is occasionally an exceptional rat whose water consumption is high. Two of these exceptions were checked up and the rats found to have a very large urine volume to account for the excess water consumed.

TABLE III.

Average Urine Output for 2 Successive Days (October 18 to 19) by Groups 56 and 57.

Female rats were used except in the cases noted.

Group No.	Fat in diet.	Rat No.	Daily urine output.	Average for group.
			cc.	cc.
56	None.	28276	3.6	
		28282	3.3	
		28281	2.1	
		28275♂	3.4	3.1
57	20 per cent lard.	28277	2.2	
		28280	4.5	
		28279	3.8	
		28278♂	4.9	3.9

To account for this difference in water consumption two values were checked: urine volume and food consumption. The measurement of urine volume was made by placing the cages over funnels and collecting the urine under toluene in 12 mm. tubes. Evaporation from the tubes was negligible but of course considerable evaporation took place on the walls of the funnels. Therefore the volumes recorded have no absolute value but should be good for comparisons. The authors were surprized to find that the average urine excretion of the animals on the fat-free diet (high water consumption) was actually less than that of the animals receiving fat. A single example of the measurements taken for 2 successive days on Groups 56 and 57 is given in Table III. Evi-

TABLE IV.

Food Consumption of Animals in Table II.

The food consumption records are taken every 4 days but the values given here are the average gm. consumed per day during each water consumption period. Female rats were used except in the cases noted.

Group No. and diet.	Animal No.	Food consumption per day.											
		Oct. 22–30.		Oct. 30–Nov. 7.		Nov. 7–14.		Nov. 14–23.		Nov. 23–Dec. 1.		Dec. 1–10.	
		gm.	*cal.**	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*cal.*
Group 3. Diet 550 B + Y† + Fraction AD (no fat).	27139	9.95	40.4	10.10	41.0	10.75	43.5	9.75	39.6	10.55	42.7	10.75	43.5
	27278	11.70	47.1	10.40	42.1	14.65	58.5	11.25	45.4	11.80	47.5	12.25	49.3
	27123	8.50	34.8	10.10	41.0	10.10	41.0	10.30	41.8	9.90	40.2	10.15	41.2
Group 4. Same + 10 drops lard.	27132	12.40	51.8	10.65	45.2	14.40	59.6	12.15	51.0	10.85	46.0	11.50	48.5
	27261	8.20	35.8	7.55	33.3	9.90	42.3	9.55	41.0	9.60	41.2	7.15	31.7
	27122	9.05	39.0	10.30	43.9	10.75	45.6	8.75	37.9	8.95	38.7	8.15	35.6
Group 19. Diet 560 B + Y + Fraction AD (20 per cent lard).	27202	9.05	45.6	9.55	48.0	10.60	53.1	8.30	42.0	9.15	46.1	9.90	49.7
	27186	9.90	49.7	9.90	49.7	13.05	64.9	10.55	52.8	10.35	51.9	10.40	52.1
	27190	6.95	35.5	8.20	41.5	8.15	41.3	8.30	42.0	8.20	41.5	8.40	42.5
Group 56. Same as for Group 3.	28276	9.05	36.9	9.90	40.2	10.75	43.5	9.00	36.7	9.95	40.4	9.40	38.3
	28282	9.10	37.1	9.90	40.2	11.80	47.5	9.80	39.8	9.65	39.3	10.50	42.5
	28281	9.60	39.1	9.45	38.5	10.25	41.6	10.00	40.6	10.50	42.5	Died.	
	28275♂	11.40	46.0	9.95	40.4	10.30	41.8	10.15	41.2	9.45	38.5	11.15	45.0
Group 57. Same as for Group 19.	28277	6.60	32.8	6.10	31.4	7.50	38.2	6.65	34.1	6.25	32.2	7.00	35.8
	28280	8.35	42.3	7.55	38.4	9.00	45.4	8.15	41.3	8.10	41.1	8.05	40.8
	28279	8.60	43.5	8.75	44.2	8.85	44.7	8.65	43.7	8.80	44.4	8.65	43.7
	28278♂	8.45	42.7	8.80	44.4	9.00	45.4	8.30	42.0	8.50	43.0	7.40	37.7

Average.												
Fat-free.	9.90	40.2	9.97	40.5	11.23	45.3	10.04	40.7	10.26	41.6	10.70	43.3
10 drops lard.	9.88	42.2	9.50	40.8	11.68	49.2	10.15	43.3	9.80	42.0	8.93	38.6
20 per cent lard.	8.27	41.7	8.41	42.5	9.45	47.6	8.41	42.6	8.48	42.9	8.54	43.2

Average for entire period.	Fat-free	10.35 gm.	41.9 cal.
	10 drops lard	9.99 "	42.7 "
	20 per cent lard	8.59 "	43.4 "

* Total calories = calories from diet + calories from supplement. Yeast has about 80 per cent digestible protein and carbohydrate; hence 0.65 gm. of yeast = 2.08 calories, 10 drops of lard = 230 mg. = 2.07 calories.

† Y designates the daily dose of 0.65 gm. of ether-extracted yeast.

dently the small animals in Group 56 (fat-free) consume twice as much water but excrete no more urine than Group 57 (20 per cent fat).

In order to determine the total water intake, the food consumption must be known for calculation of the water of metabolism. Food consumption is given in gm. and calories in Table IV. The average figures show that the calorie intake is practically identical for all groups, the difference between the fat-free group and those animals receiving some fat being only 1 calorie in 42, or less than 2.5 per cent. This probably is not significant. Since the water value of a calorie of these diets is only about 0.14 gm., the lesser requirement for water by the normal animals cannot be accounted for by greater food consumption.

Summarizing, we find that animals receiving no fat consume almost twice as much water as their controls receiving fat and that they do not excrete this excess water as urine. The feces are very dry and are very small in amount. Although they weigh only 80 per cent as much as their controls, the animals on the fat-free diet consume the same amount of food. They are not hyperactive. The excess water used (10 gm. daily) must be lost by evaporation from the lungs and skin. This alone amounts to nearly 6 calories, or 14 per cent of the total calorie consumption. Further, it has been pointed out by Du Bois (5) that thin people may produce 50 per cent more heat per kilo of body weight than fat people. The rats on the fat-free diet are very emaciated and it seems that the larger water loss and lack of body fat may readily account for the large food consumption of such small animals.

This indicates that the careful collection of food consumption data may be almost worthless unless the water loss and amount of fat on the animal are taken into consideration.

The water loss by evaporation reflects in some way the condition of the skin or lungs or both. It is not known which is involved but it is quite clear that the lack of dietary fat has so injured the tissues that they are no longer the normal membranes separating the interior of the animal from its relatively dry air environment.

Effect of Fat Exclusion on Ovulation and Reproduction.

As was pointed out in our earlier paper it was thought desirable to exclude all materials not known to be essential to the well being

of the animals. It has been known for some years that both males and females thrive on a diet deficient in vitamin E. Accordingly this vitamin was not included in the diet of our first groups of animals. But it has been recently pointed out by Evans (6) that rats attain a greater size when vitamin E is included in the diet. In order to exclude the vitamin E factor from the new disease and to demonstrate that none of the effects noted on low fat diets could be ascribed to the lack of vitamin E (*i.e.* poor growth, diseased skin and kidneys, increased water consumption) a concen-

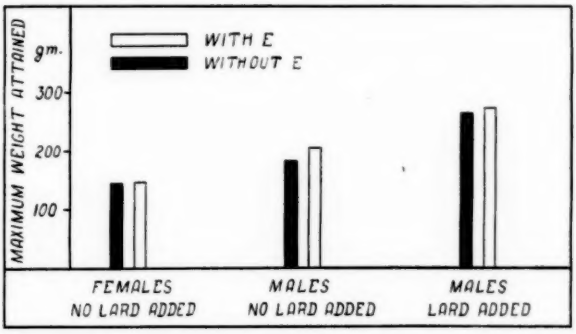

CHART 1. Average maximum weights attained by male and female rats without the addition of Fraction E to fat-free diets and to diets supplemented by 10 drops of lard. Total number of males, 22; total number of females 22.

trated, fat-free preparation called Fraction E has been added to the daily yeast dose of all animals started during the past year.

The preparation of Fraction E is identical with that of Fraction AD (1). Well cleaned wheat germs from the Washburn Crosby Milling Company are extracted with purified U.S.P. ether in a large Soxhlet apparatus (1 gallon size). The last of the ether is removed from the oil *in vacuo* at 60°. The oil is stored at 0° in a dark bottle.

For a group of 60 rats, 30 gm. of wheat germ oil are saponified with 20 per cent alcoholic KOH, and the non-saponifiable matter is extracted with 300 cc. of U.S.P. ether (for anesthesia) and washed free from alkali. The ether is concentrated to 20 cc. and a drop or two evaporated on the yeast dose daily. This quantity is fed

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every 2 weeks. Therefore each animal receives the non-saponifiable matter from 250 mg. of wheat germ oil each week. It is known that 250 mg. of a good wheat germ oil will cure a sterile female, so that this amount fed each week was expected to be entirely adequate. Our fertility tests have proved this to be true.

We have made no effort to determine finally whether vitamin E has any appreciable effect upon the growth of animals on a fat-free diet. When the effects are small and the variations are large, more animals are required than we have been able to give to prove this one point. From Chart 1 it is evident however that in these two groups of twenty-two females (total) there is no appreciable effect due to vitamin E. These animals were selected litter mate sisters, reared especially for this comparison.

No comparable experiment has been performed with males. Since the lack of vitamin E causes a degeneration of the seminiferous epithelium of the testis, it might be suspected that tissues which affect the growth of the male would also be somewhat affected. We have recently reared a group of seventeen males with Fraction E. For comparison with these there are only four males reared a year earlier. Groups so widely separated in time should not ordinarily be compared. But the great uniformity of the diet, colony temperature, and cage conditions may justify the present comparison.

The average maximum weight attained by the seventeen males receiving Fraction E is compared with the average maximum weight of the four males without Fraction E in Chart 1. On the same chart are plotted average maximum weights of males receiving 10 drops of lard without Fraction E and 10 drops of lard with Fraction E. This limited evidence indicates that the males receiving no fat were helped more by the addition of Fraction E than those receiving fat. This might be interpreted as meaning that the non-saponifiable matter fed with Fraction E is helpful and this same non-saponifiable matter is fed with the vitamin E-free lard. But in no case is the effect of vitamin E large as compared with the effect of a trace of fat in the diet. Furthermore, all animals receiving vitamin E develop the scaly condition and kidney trouble as early as those animals without vitamin E. We feel justified in concluding that vitamin E offers no protection from the new disease.

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Ovulation of the twenty-two females compared in Chart 1 may serve as another check on the effect of vitamin E on the general well being of females receiving a fat-free diet. They may be divided into the groups: (1) those ovulating regularly (every 4 to 6 days), and (2) those ovulating very irregularly (less than every 9 days) or not at all. Comparative data for the two groups are as follows:

Total No. of rats.	Addition to Diet 550 + Fraction AD + yeast.	No. ovulating regularly.	No. not ovulating regularly.
11	+ Vitamin E	4	7
11	− " "	5	6

It is evident that vitamin E does not affect the well being of the females on the fat-free diet sufficiently to improve ovulation.

It should be pointed out here that in the study of the effects of the well known vitamins carried in cod liver oil and wheat germ oil the results may be confused by the use of the whole oil rather than just the non-saponifiable fraction. 3 drops daily of cod liver oil or of wheat germ oil will cure badly diseased animals when these animals are suffering from a lack of essential fatty acids. Many of the ordinary diets used in nutrition may be deficient in this respect.

Although Fraction E has no effect on ovulation, curative oils cause an immediate resumption of ovulation in those females in which ovulation has ceased. The figures cited above show that in less than 50 per cent of that special group was ovulation regular. About half of these animals had not ovulated at all. The animals which are not ovulating at all provide fine material for testing the effect of fats on ovulation. Four examples are given in Table V. Those animals which received 5 drops daily of corn oil, olive oil, or linseed oil ovulated within 5 days after the dose was begun; *i.e.*, vaginal smear changes were resumed as quickly as though they were castrated females receiving a daily injection of ovarian hormone. On the other hand, the coconut oil, which is not a curative oil, did not cause the resumption of ovulation.

It is an almost invariable rule that every animal which is receiving a curative oil and has resumed growth also ovulates normally (a cycle every 4 to 6 days). We need not interpret this as meaning

TABLE V.

Immediate Resumption of Normal Ovulation by Females, in Which Ovulation Had Ceased, on Low Fat Diet, When Fed 5 Drops Daily of Curative Oil.

Date.	Ovulation histories.			
	Rat BH29537.	Rat B29543.	Rat W29544.	Rat W29535.
1919				
July 1	LE	LE	LE	LE
" 2	"	"	"	"
" 3	"	"	"	"
" 4	"	"	"	"
" 5	"	"	"	"
" 6	"	"	"	"
" 7	"	"	"	"
" 8	"	"	"	"
" 9	"	"	"	"
" 10	"	"	"	"
" 11	"	"	Linseed oil begun.	"
" 12	"	"	LE	"
" 13	Corn oil begun.	Olive oil begun.	"	Coconut oil begun.*
" 14	LE	LE	"	LE
" 15	"	"	"	"
" 16	"	"	"	"
" 17	"	" ?	0–1	"
" 18	0–1	Cornif.	Cornif.	"
" 19	Cornif.	All three.	All three.	"
" 20	"	LE and C.	LE and C.	"
" 21	All three.	0	" ?	"
" 22	LE and C.	Cornif.	0–1	"
" 23	"	All three.	Cornif.	"
" 24	0–1	" "	All three.	"
" 25	Cornif. and leuc.	0–1	LE and C.	"
" 26	All three.	Cornif.	"	"
" 27	LE and C.	Cornif. and leuc.	0–1	"
" 28	" " "	LE and C.	Cornif.	"
" 29	0–1	"	LE and C.	"
" 30	Cornif.	" ?	" " "	"
" 31	All three.	Cornif.	"	"
Aug. 1	LE and C.	All three.	Cornif.	"

The stages in the estrous cycle are indicated as follows: 0 = epithelial cells only, in heat toward end; 0–1 = epithelial and cornified cells, in heat; cornif. = cornified cells only, possibly still in heat; cornif. and leuc., all three, and LE and C = varying mixtures of cornified cells, leucocytes, and epithelial cells, not in heat; LE = diestrus with varying amounts of leucocytes, epithelial cells, and mucus.

* Coconut oil does not cure readily.

that these oils contain a fatty acid specific for ovulation. Ovulation may be considered as dependent upon the general well being of the animal, a function which suffers when the animal is in poor condition or its metabolism is low. The added oils so improve the animal as a whole that normal ovulation is resumed.

But much work indicates that the ovarian hormone is a lipoid and certainly its method of extraction shows it to be closely associated with the cell lipoids. Furthermore, the resumption of ovulation is so rapid that growth has hardly begun. Because of these facts, as well as the effects of fat upon the male (see Table VII), we are inclined to the view that the synthesis of ovarian hormone ceases when fatty acids are eliminated from the diet because the fatty acids are closely associated with the hormone.

Fertility.

As a test of the effectiveness of our vitamin E preparation three females which were on the fat-free diet were bred to normal males. These females were the only ones of the group which were ovulating. They were bred in wire bottom cages, with access to no food except their usual pure diet. They were then returned to their individual wire bottom cages for gestation. Vitamin B was not increased. It was hoped that the young would fall through the large wire mesh and not be destroyed. Data for the actual gestations are given in detail (Table VI) so that the gain in weight, prolonged presence of red blood cells, and delayed littering may be clear. Two of these three very emaciated animals actually produced litters, proving the presence of sufficient vitamin E in their diets. Although they had been on a growth plateau for over 6 weeks, presumably due to the limitations of their diet, they gained 12 to 17 gm. during gestation. None of the young lived more than a few hours after birth.

The tremendous effect of the fatty acid fraction of cod liver oil and of wheat germ oil may be best illustrated by gestation studies. Six females on Diet 550 B were changed from the non-saponifiable matter, Fraction AD, to the 2 drops of cod liver oil daily. In 4 weeks their weight had increased markedly (an average of about 35 gm. per animal). All were ovulating and were mated positively. On the day of positive mating 250 mg. of wheat germ were added to the daily supplement. One of the six animals (Rat 27169)

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TABLE VI.

Gestation Histories of Three Females (from Group 62) on Fat-Free Diet 550 B + Fraction AD + Fraction E.

Date.	Rat BH29545.		Rat BH29538.		Rat BH29539.	
		Weight.		Weight.		Weight.
1929		*gm.*		*gm.*		*gm.*
June 26	0–1 mated with ♂ W29526.	143				
" 27	Cornif., plug, sperm.					
" 28	LE and C.					
" 29	LE	144			0 mated with ♂ 29509.	168
" 30	"				Cornif., plug, sperm.	
July 1	"		0–1 mated with ♂ 29526.	137	" and leuc.	
" 2	"		Cornif., plug, sperm.		LE and C.	
" 3	"		All three.		LE	
" 4	"		LE		"	
" 5	"		"		"	
" 6	"	150	"	139	"	170
" 7	"		"		"	
" 8	"		"		"	
" 9	"		"		"	
" 10	R.B.C. (light).		"		"	
" 11	" (very heavy).		"		R.B.C. (faint).	
" 12	Vagina full of dark red blood; not normal.		"		" "	

July 13	R.B.C. (very heavy).		LE		R.B.C.	175
" 14	" " "	155	"	142	" (heavy).	
" 15	" " "		R.B.C. (very heavy).	141	" (faint).	
" 16	"		" " "		" (heavy).	
" 17	LE, isolated.	153	" " "		LE	
" 18	R.B.C. (faint).		"		R.B.C.	185
" 19	No litter.	153	" (faint).		" (faint), isolated.	
" 20	" "	152	" "	149	No litter. R.B.C.	181
" 21	" "	153	" " isolated.		" " "	183
" 22	" "	149	No litter.	149	" " " (heavy). Large blood spot under cage.	
" 23	" "	149	" " R.B.C. (faint).		R.B.C. (heavy). Litter. 1 ♀, 3.4 gm.	
" 24	" "	151	" "	153	Littering continued. 1 ♀, 4.5 gm., partly eaten; 1 ♀, 4.5 " " " ; 1 ♀, 4.5 " still alive.	150
" 25	Cornif.	149	" " R.B.C. (faint).			
" 26	LE and C.		" " " "			
" 27			Litter, 1 dead, partly eaten; 1 dead, body eaten.	149		

0 = epithelial cells only, in heat toward end; 0–1 = epithelial and cornified cells, in heat; cornif. = cornified cells only; cornif. and leuc. = cornified cells and leucocytes; LE = diestrus; R.B.C. = red blood cells which appear about the 14th day of a normal gestation and disappear within 2 or 3 days. The presence of red blood cells proves that positive fertilization and implantation have taken place.

became wheezy and sick and failed to gain any weight. Resorption occurred and the animal died 2 weeks later. The other five animals (Rats 27181, 27259, 27176, 27182, 27154) had an average gain of 55 gm. during gestation as compared with 12 to 17 gm. for the three animals receiving Fraction AD + Fraction E instead of 2 drops cod liver oil + 250 mg. of wheat germ. Rat 27181 had a litter of only two (females), weighing 5 gm. each. These were eaten the 2nd day. The other four rats produced four litters, totaling twenty-five young, with an average weight of 5.3 gm. The young were all lactated successfully with no loss of weight by the mothers. The weaning weights were small (average weight = 29 gm. on 21st day) but the young were in good condition.

Although this vitamin E preparation keeps the healthy animals which receive fat fertile, in order to prove that the vitamin E was actually retained by the animals on low fat diet the above test was necessary. It appears that we are justified in concluding that the vitamin E was absorbed in the absence of the dietary fat and that the very poor gestations were due to the poor condition of the females.

It seemed that males might be more useful than females for testing this point. Their tests simply show whether or not they are capable of mating and fertilizing the eggs of normal stock females, without the uncertainty as to whether failure in gestation is due to a lack of vitamin E or to the general poor condition of the animal. Two groups of males have been thus tested (sixteen in one group and ten in another). Normal young stock females, showing the 0 or 0 to 1 stage of the ovulation cycle, were put with the males and examination was made the next morning for plug and sperm. If a positive mating took place the gestation was followed in the usual way, except that daily smears were taken. Each male in the group of sixteen animals was given four chances to mate, unless a positive mating resulted sooner. Only normal females were used. That is, if a female failed to show positive fertilization after a positive mating, the animal was tested with a normal stock male. Each male of the group of ten was given six chances to mate unless positive mating occurred sooner. The results are summarized in Table VII. The difference between those males receiving 10 drops of lard and those receiving no fat is remarkable. A new and uniform cause of sterility is shown. The

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normal sex responses have been lost in most cases, while in vitamin E sterility the sex responses are retained long after the loss of the

TABLE VII.

Comparison of Mating Histories of Male Rats Which Received 10 drops of Lard with Those of Males on Complete Fat-Free Diet 550 B + 0.65 gm. Yeast + Fraction AD + Fraction E.

Total attempted matings = the total number of females in estrus put with the males of each group. Total positive matings = total number of females which showed plug and sperm (or just sperm) the day after mating. Total R.B.C. = total number of positive fertilizations.

Group No. and diet.	Total No. of rats.	Age.	Average weight.	Total attempted matings.	Total positive matings.	Total R.B.C.	Total litters.	Per cent of males proved fertile.
		mos.	*gm.*					
Group 58. Diet 550 + Y* + Fraction AD + Fraction E.	5	5–6	202	17	7	0	0	0
Group 59. Diet 550 B +Y+ Fraction AD + Fraction E.	6	5–6	199	22	0	0	0	0
Group 65. Same as for Group 59.	5	4	147	30	0	0	0	0
Group 60. Same as for Group 58 + 10 drops lard.	2†	5–6	255	5	1	1	1	50
Group 61. Same as for Group 59 + 10 drops lard.	2	5–6	251	2	2	2	2	100
Group 63. Same as for Group 61.	5	4	226	10	6	5	5	100

* Y designates the daily dose of 0.65 gm. of ether-extracted yeast.

† Rat 29541 was a nervous, abnormal animal which refused to mate in four attempted matings. The other, Rat 29025, mated the first time.

seminiferous epithelium (6). The testis is small and watery but the histology is not yet known. The animals were small but their condition was fairly good at the time of the tests (4 to 6 months

old). Yet the effect on the animal is so profound that in only three cases were there positive matings and in no case was there a litter sired. Of the nine animals receiving 10 drops of lard, eight sired normal litters and the ninth animal was nervous and somewhat abnormal in general behavior.

It is impossible to know from this experiment whether vitamin E was absorbed by the animals on the fat-free diets, while it is certain that the animals receiving 10 drops of lard received a sufficient supply of vitamin E. Those three animals in the high protein group which showed a total of seven sterile matings may be sterile from a lack of vitamin E. Even if this proves to be true, the sterility must nevertheless be considered as primarily due to the lack of fat in the diet since the same quantity of vitamin E in the presence of 10 drops of lard is adequate. The restoration of the normal sex responses by the addition of lard to the diet is now being tried.

The testicular hormone, which is responsible for the development of secondary sex characters and possibly responsible for the normal sex responses, is also a lipoid if the solubility of the impure substance is a correct indication. Its purification can be effected by very much the same procedure as that used for the ovarian hormone (7). It is possible that the almost complete loss of sex response by the males on the low fat diet is due to the failure of this hormone to be synthesized in the absence of the essential fatty acids.

Nature of Essential Fatty Acids.

Since the new disease is caused by a lack of the fatty acids present in lard, the problem of the comparative value of the acids known to be in lard was next attacked. It is well known that the preparation of pure fatty acids is quite difficult. But a greater drawback to the use of individual fatty acids is found in the isomeric changes of the unsaturated acids when isolation is attempted. All workers recognize the fact that the acids isolated by the bromination method may not have exactly the same structure that they had in the natural oil. Therefore, a series of oils was chosen which would give a variety of fatty acid combinations, as shown by the best available analyses. On the basis of the results of Ellis and Isbell (8) it was assumed that the pig can readily synthesize palmitic, stearic, and oleic acids from carbohydrates but

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that linolenic and linoleic acids may be entirely of dietary origin. Arachidonic acid did not show such a definite trend but the results indicate that it may be largely of dietary origin.

The best available analyses of a selected group of fats and oils used in curative work are assembled in Table VIII. Fortunately we are able to get two fats (coconut oil and butter) of high digesti-

TABLE VIII.

Approximate Fatty Acid Content of Oils and Fats Used for Cures.

Butter fat and lard probably vary most widely from these figures.

	Coconut oil (9).	Butter fat (10).	Olive oil (11).	Lard (8) (corn-fed hogs).	Corn oil (12).	Poppy-seed oil (13).	Linseed oil (14).
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Unsaturated acids.							
Oleic.	5.5	31.1	80.5	52.0	45.4	28.3	4.5
Linoleic.	1.3	0.0?	6.9	6.7	40.9	α29.5	α17.0
						β29.0	β41.8
Linolenic.	0.0	0.0	0.0	0.0	0.0	0.0	α20.1
							β 2.7
Arachidonic.	0.0	0.0	0.0	0.06	0.0	0.0	0.0
Saturated acids (total).	83.4	63.7	11.2	36.9	11.8	7.2	8.3
Lignoceric.					0.2		
Arachidic.			0.2	0.0	0.4		Trace (16).
Stearic.	3.0	11.4	1.4	12.2	3.5	Present (15).	Little.
Palmitic.	7.6	15.5	9.4	24.1	7.7	"	Present.
Myristic.	18.5	22.6	0.2	0.6		"	"
Lauric.	50.3	6.9				"	
Soluble (total).	4.0	7.3					

bility which contain little or no linoleic acid and no more unsaturated acids. Olive oil, lard, corn oil, and poppy-seed oil furnish increasing amounts of linoleic acid without any linolenic acid. Linseed oil is rich in linoleic and linolenic acids with very little oleic acid. Lard is peculiar in having arachidonic acid instead of arachidic acid which is present in most plant oils. The coconut oil, olive oil, lard, and corn oil were well known brands of refined

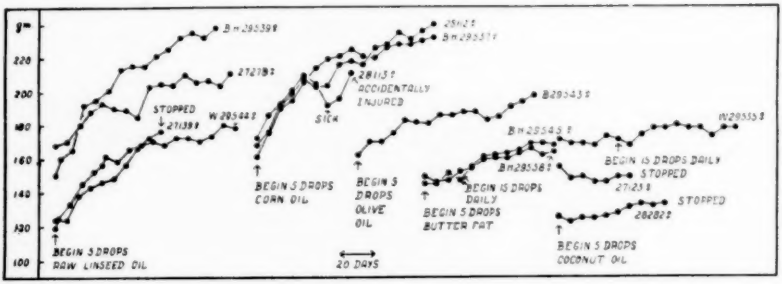

Chart 2. Weight curves showing the growth response of cures to the addition of five natural oils to the fat-free diet. Rats receiving linseed oil, corn oil, or olive oil were cured of scaly feet and dandruff on the back. Rats receiving butter fat or coconut oil showed no improvement of the skin.

products on the local market. Poppy-seed oil was purchased from Eimer and Amend. The raw linseed oil was obtained from a paint company. Butter fat was made by melting the highest grade butter in a moderately warm water bath.

As in previous work, the tests were made by the cure method. Young animals were put on the fat-free diet at weaning and after a few months growth was stationary, they had scaly feet and dandruff on the back, and were consuming more than normal quantities of water. Diet 550 B was used from the day of weaning so that growth would stop and the scaly condition supervene with as little degeneration of the kidneys as possible. The animals were then given the curative dose on yeast. The results of the first series of tests are shown in Chart 2. The older work with 10 drops of lard is not included (see (1) p. 363). The results are so clear cut that no extended discussion is necessary. Butter and coconut oil which contain very little linoleic acid and no more unsaturated acids allow little or no growth when fed at the 5 drop level and cause no clearing of the scale or dandruff. Olive oil takes an intermediate position, while corn oil and linseed oil are about equally good. The results with butter are of particular importance since the butter adds appreciable amounts of vitamins A and E to the diet without improving the animals' condition.

An examination of the composition of these oils in Table VIII leads to the conclusion that linoleic acid is the essential unsatu-

rated fatty acid, with the possibility that arachidic acid may also be involved. The failure of coconut oil might be attributed to its low content of C_{18} acids, which would not give the liver a chance for desaturation. This point was tested in the next group of experiments.

To measure again the relative value of saturated acids, oleic acid, and linoleic acid, it seemed desirable to have a readily digestible fat of complex composition but containing no unsaturated acids. Ozaki (17) has shown that the chief fatty acids of coconut oil, lauric and myristic, are equal to oleic acid in digestibility and growth-promoting power. Furthermore, coconut oil may be completely saturated by hydrogenation and still have a low melting point. This makes the hydrogenated oil an ideal basal fat. This product was very kindly furnished us by Dr. J. J. Vollertsen, Chief Chemist, Armour and Company. It was prepared under his direction especially for this work. The hydrogenated oil had the following constants: free fatty acids 0.04 per cent, iodine number 0.61. It melts gradually over a wide range and is completely liquid at 50°. Since all of the oleic acid and linoleic acid has been converted into stearic acid, the total stearic acid of the hydrogenated oil is 9.8 per cent.

Ozaki (17) has further shown that although the free fatty acids are generally poorly utilized the methyl and ethyl esters are well utilized. This is especially true of the saturated fatty acids. It was decided, therefore, to feed the individual fatty acids as their methyl esters. Methyl stearate (Eastman, MP 36–38°) was used as a source of stearic acid.

Methyl linolate was prepared by the method of Rollett (18) from the same sample of corn oil as was used for the earlier feeding experiment. Corn oil contains no more highly unsaturated acid so that an uncontaminated tetrabromide is obtained. The properties of the debrominated methyl ester agreed with those given in the literature. Rollett showed that this acid is not homogeneous but is a mixture of more or less definite proportions of α and β forms. There may be even more than two isomers present. But it was hoped that the linoleic acid thus prepared would have a considerable proportion of the acids as found in natural plant oils.

Two more animals were given 15 drops of butter fat daily to

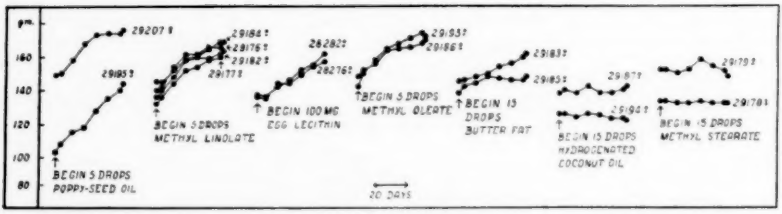
BEGIN 5 DROPS POPPY-SEED OIL
BEGIN 5 DROPS METHYL LINOLATE
BEGIN 100 MG. EGG LECITHIN
BEGIN 5 DROPS METHYL OLEATE
BEGIN 15 DROPS BUTTER FAT
BEGIN 15 DROPS HYDROGENATED COCONUT OIL
BEGIN 15 DROPS METHYL STEARATE
20 DAYS

CHART 3. Weight curves showing the growth response of cures to some oils and special fatty substances. Poppy-seed oil and methyl linolate completely cured the scaly skins. Methyl oleate and egg lecithin partially cured the skin. Rats receiving butter fat, hydrogenated coconut oil, or methyl stearate showed no improvement of the skin.

check again the value of oleic acid. Since butter fat contains a minimum of 30 per cent oleic acid, the 15 drops (averaging 330 mg.) contributed 99 mg. of oleic acid to the daily diet.[4] This is about 1 per cent of the total food consumption. As a further test of oleic acid 5 drops daily of methyl oleate (Eastman, Practical, BP 189–191°/10 mm.) were fed to two other rats.

Another oil, poppy-seed oil, containing no acids more unsaturated than linoleic, was added to the list.

Another source of fatty acids which can be freed from most contamination is lecithin. The fatty acids of lecithin are mixed, possibly as badly mixed as they are in a whole oil, but the lecithin can be so purified as to dispel any illusion that the cure of the disease is due to "vitamins" or other indefinite substances mixed with the fatty acids. Egg yolk lecithin was prepared according to the method of Levene and Rolf (19). This lecithin is free of amino nitrogen and has the correct analysis except for a slightly high nitrogen content. Egg yolk lecithin contains the following acids: palmitic, stearic, oleic, linoleic, and arachidonic. The presence of linolenic acid is uncertain (20).

The results of feeding these substances are summarized in

[4] Medicine droppers of uniform tip have been used throughout this work. The following average values of the drops have been found: 10 drops lard = 232 mg.; 10 drops butter fat = 218 mg.; 10 drops hydrogenated coconut oil = 197 mg.; 10 drops methyl stearate = 185 mg.; 10 drops methyl linolate = 180 mg.

Chart 3. These tests confirm our earlier findings (Chart 2) that the curative effect of oils is due largely to acids more unsaturated than oleic acid. And the fairly good gains made on pure methyl linolate indicate that linoleic acid is highly important. Furthermore the rats receiving linoleic acid lost the scale and dandruff from their feet and backs. Failure to grow on 5 drops of butter followed by only slight growth on 15 drops upholds the view that the oleic acid in butter is not of great value as a cure for this disease. However, butter probably contains some linoleic or other unsaturated acid not precipitated by the ordinary analytical methods (21). If this is true, then the better growth of the rats on larger doses of butter fat is readily explained; otherwise we must assume that oleic acid is of some value. It should be pointed out, however, that even 15 drops of butter fat do not cure the scaliness of the feet and back. The butter causes a return to the condition found in the early life of the animal, slow growth taking place, while the feet and back are very scaly.

Since lecithin did not bring about a more rapid recovery than did the methyl esters of fatty acids, it seems quite clear that the animals do not need some special lipoids but only the fatty acids. It is true that the word lecithin covers a group of substances of variable composition and it is quite possible that other lecithins are more valuable than egg lecithin. Liver lecithin is now being investigated.

The high curative value of commercial methyl oleate is to be attributed to the presence of more unsaturated acids. Commercial oleic acid is made from olive oil which contains 7 per cent linoleic acid. In the preparation of oleic acid no effective methods are used to remove the linoleic acid present. A considerable amount of methyl linolate will distil with larger amounts of methyl oleate. In fact, on bromination in petroleum ether an appreciable precipitate was obtained and we know that the solubility of the tetrabromide in the presence of the dibromide is fairly high.

We must then conclude that this small amount of linoleic acid in the presence of oleic acid is almost as effective as a much larger amount of pure linoleic acid, while oleic acid alone is of much smaller value. Otherwise we would be forced to the conclusion either that the oleic acid of butter is different from that prepared from olive oil or that the other fatty acids of butter are actually

detrimental. These points are now being studied with larger numbers of animals.

Finally, the data show conclusively that the saturated acids of coconut oil and methyl stearate will not promote renewed growth or cure the skin, although they have high food value.

Chart 4 summarizes the average maximum gains made by the rats in 40 days after the curative doses were begun. All of the groups in Charts 2 and 3 are included. The average for the rats

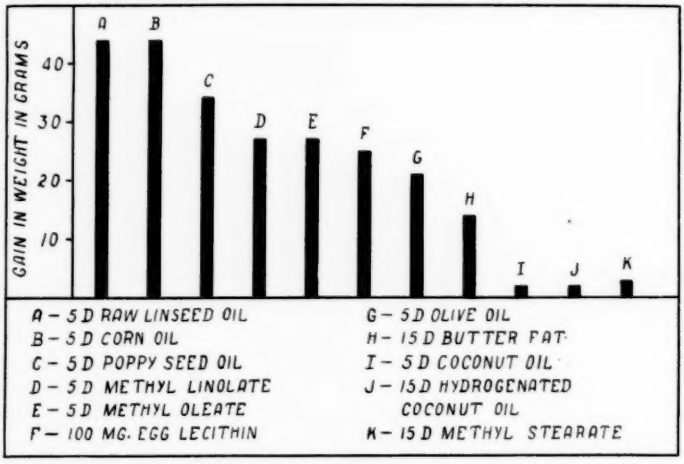

CHART 4. Mean maximum gains made in 40 days after feeding 5 to 15 drops of cure lipoids. Data from Charts 2 and 3.

receiving 15 drops of butter includes the data from both charts 2 and 3. It should be pointed out that the growth response to butter was due largely to the high level at which it was fed. When fed at the 5 drop level, the animals did not grow (Chart 2).

DISCUSSION.

Recently considerable emphasis has been placed on the fat requirements of the rat and mouse. Although the earlier work indicated that a fat-free diet was adequate in every respect (22, 23), the later work has not been in agreement with this point of

view. Hesse (24) found weight increases and increased oxygen consumption of mice on a bread diet after addition of fat and phosphatides. Wesson (25) found some interesting effects of the addition of certain fats to the diet. Jaffé (26) found that mice could not be supported on a diet of white bread, beef, and synthetic fat without the addition of a mixture of lipoids called Promonta. But the three works above were concerned especially with more complex lipoids rather than with fatty acids. Furthermore, the diets were not always balanced or the fat-soluble vitamins were not certainly present so that interpretation of the results cannot well be made. In 1928 Evans and Burr (27) showed a definitely subnormal weight and irregular ovulation on diets complete in every respect except for fats. Last year Burr and Burr (1) found that a definite deficiency disease resulted from the rigid exclusion of fats from the diet. At about the same time McAmis, Anderson, and Mendel (28) reported subnormal weights for animals on a low fat diet, thus supporting the view that fats are beneficial to the rat. Palmer and Kennedy (29) did not find fat beneficial but their control diets were not fat-free. 160 to 500 mg. of cod liver oil were fed daily and this amount is entirely adequate to protect against the low fat disease. Evans and Lepkovsky (30) have suggested that the beneficial effect of fat in the diet is apparent only when the antineuritic vitamin is low. But their work is not a critical check on the effects of small amounts of fat in the diet since they also fed 2 drops of cod liver oil daily along with several drops of wheat germ oil. The deficiency disease reported by Burr and Burr (1) occurs regularly when an excess of vitamin B is fed (0.65 gm. of dry whole yeast daily) and is not cured by an increase of 30 per cent in the yeast dose. The present authors showed in their earlier paper that the fatty acids were the essential part of the fat and that those acids in lard were adequate. In the present paper it has been shown that only the unsaturated acids will cure the sick animal and that pure linoleic acid is far better than the oleic acid of butter.

The finding that saturated fatty acids do not cure the low fat disease or promote renewed growth while certain unsaturated fatty acids do, leads to the conclusion that this work is not concerned with the fat minimum of Krogh and Lindhard (31). Their conclusions were based upon quantitative data, and all fats of respira-

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tory quotient 0.71 and equal digestibility would be of equal value. But for the present work fats are not of equal value and the differences are due to the structure of the fatty acids. We have thus taken another step away from Rubner's law of isodynamic equivalence, and chemical structure in the fats has assumed some of the same importance as in the proteins (32). For the rat, a typical warm blooded animal, unsaturated fatty acids are essential constituents of the diet.

Evidence has been presented to show that oleic acid as found in butter and coconut oil is of little value, while linoleic acid, in oils and isolated, is of great value in the diet. Linolenic acid and arachidonic acid are now being studied in isolated forms. Ordinary arachidonic acid does not occur in the oils of highest curative value (corn oil and linseed oil) in our series. Thus it would seem to be not essential in the diet. But the importance of arachidonic acid as an intermediate in metabolism has been emphasized recently by Wesson (33). This importance has been hypothetical, based upon the indirect evidence of its occurrence in various tissues. Certainly the arachidonic acid content of active tissues such as liver, pancreas, kidney, suprarenal, and spleen is high (33, 34) and it is natural to assume some important rôle for this highly unsaturated, long chain acid.

In the absence of complete analytical data (which we are now collecting) no exact statement can be made as to the rate of fat synthesis in the presence of small amounts (100 mg. daily or less) of unsaturated fatty acids but this is of such interest that it should be mentioned here. The small emaciated animals on the fat-free diet consume as much food daily as their larger, fat-containing controls (Table IV), yet this food is burned and none is used in growth and fat synthesis. On the addition of the unsaturated fatty acids, growth is resumed and a normal amount of subcutaneous and visceral fat is found in the cured animal at autopsy. Therefore, fat synthesis is dependent upon the presence in the tissues or blood stream of a certain minimum quantity of the essential fatty acids. What percentage of the growth observed is due to storage of fat and what percentage is due to increase in other compounds (protein and carbohydrate) is not yet known.

This brings up the interesting question of desaturation by the liver. Since the work of Leathes (35) it has been generally ac-

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cepted that the liver readily desaturated all the fatty acids necessary for the organism's needs. But the careful work of Ellis and Isbell (8) points to the view that the ability of the warm blooded animal to produce unsaturated fatty acids may be quite limited and that oleic acid represents the greatest degree of unsaturation attainable over long periods of time. In discussing these results Bloor stated, "the extent of the ability of the liver to desaturate is not known and it is possible that the ability of the animal body to synthesize fatty acids may be limited" (36). The results of Ellis and Isbell are of course concerned only with the rendered lard and do not give us values for all tissue fats. But other feeding tests indicate that most animal fats become more saturated the freer the diet is from unsaturated acids. McAmis, Anderson, and Mendel (37) fed rats a high sucrose, fat-free diet and rendered the fat of the entire animal. This fat had an iodine number of 64 to 71, a fairly normal value for lard. In a more extensive paper Eckstein (38) showed that the rat produced fat of about the same iodine number and saponification number from diets high in carbohydrate or high in protein. The iodine values ranged from 62 for the skin to 74 for the organs. It would seem, then, that the rat, like the pig, tends to synthesize a relatively constant body fat which contains very little of any acid more unsaturated than oleic. Eckstein's work shows this very clearly in the case of arachidonic and linoleic acids. Although the total fat of young rats (less brain and gastrointestinal tract) at weaning (40 gm. in weight) contains 1.06 per cent arachidonic acid and 3.21 per cent linoleic acid, after the test animals had been kept on a low fat diet for 8 weeks these values had fallen to 0.12 per cent and 0.31 per cent respectively. Linoleic acid thus reached a much lower value than that found for lard from pigs on brewers' rice (8).

Butter fat has been carefully studied by Holland and coworkers (10). Butter production represents a long continued, high rate fat synthesis from materials in the blood stream and Holland *et al.* have found that although the type of fat produced is not affected by the protein-carbohydrate ratio of the diet, the addition of oils to the diet has a very material effect. Their cows were kept on a hay-grain basal ration containing about 2.5 per cent extractable fat (composition unknown). Cows on this diet produced a butter fat of low iodine number (about 28) and low oleic acid content

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(30.7 per cent). On adding 0.75 pound of coconut oil daily no appreciable change in iodine number or oleic acid content took place, but when like quantities of peanut oil, corn oil, or soy bean oil were fed, the iodine number rose and the oleic acid content increased to 42 to 46 per cent. Linoleic acid was not definitely determined to be present or absent. It seems quite definitely demonstrated that butter fat produced *de novo* by the cow from non-fats probably would contain a definite minimum of oleic acid (about 30 per cent) and no more unsaturated acids. Maynard and McCay (39) have recently given evidence supporting this view.

Finally, there is the much earlier work of McCollum, Halpin, and Drescher (40) which was concerned with the phospholipids of hen's eggs and is therefore of even more vital interest. The production of egg yolk by hens is another case of long continued and rapid lipoid synthesis. The above workers showed that when hens are maintained on a low fat ration the iodine number of the phospholipid falls from a normal of 63 to 34, while for the ordinary fats it falls from 64 to 52. The phospholipids are more seriously affected than the ordinary fats, and again it would seem that another warm blooded animal is definitely limited in its ability to synthesize highly unsaturated fatty acids.

But the degree of this limitation was hardly suspected until the results of the present paper were obtained. It has been definitely shown that when a rat has been reared on a fat-free diet its store of unsaturated acids has been so depleted that its health suffers severely. And the feeding of 15 drops (3 per cent of the total diet) of the mixed fatty acids of hydrogenated coconut oil or of a like amount of methyl stearate does not make possible the production of the required unsaturated acids. The actual minimum of linoleic acid required, as indicated by the response to lard, corn oil, methyl linolate, egg lecithin, and impure methyl oleate, is so small that it seems probable that the liver of these animals is unable to produce any linoleic acid. Our working hypothesis is, therefore, that warm blooded animals in general cannot synthesize appreciable quantities of linoleic acid and some of the other highly unsaturated acids. Furthermore, it is assumed that these unsaturated acids are normal constituents of essential cellular phospho-

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lipids and that the more active the tissue the greater the requirement for the unsaturated phospholipids (36, 41).

This conclusion does not reject the work of Leathes. It must be remembered that he observed apparent desaturation of ingested oils by normal livers. The two results are harmonized by either of the following conditions: (1) desaturation of oleic acid does not produce linoleic and linolenic acids but produces other substances which absorb iodine; or (2) the presence of undetermined acids is essential to the desaturation process and animals long deprived of such acids lose this function of the liver. The first condition is illustrated by the production of hepatic oleic acid discovered by Hartley (42).

Whichever hypothesis is correct, the end result is the same. The supply of unsaturated acids in the animal is soon depleted and the tissues begin to suffer. Oils are reputed to have a beneficial effect upon the skin and we find that the skin soon becomes scaly in the absence of the unsaturated acids. The other organ which apparently suffers most is the kidney. This result is to be expected when we remember that the kidney is the most active of semipermeable, secretory membranes and that membrane structures contain intimately associated lipoids (43). As shown by McCollum, Halpin, and Drescher, even the phospholipids may become more and more saturated. Hydrogenated lecithin differs markedly from ordinary egg lecithin. The amount of lecithin might remain quite constant, while its value as a membrane constituent would be lost. Furthermore, oxidation activity has been ascribed to the kidney in addition to its membrane activity. Much work has been done to show that the highly unsaturated acids may play an important rôle in biological oxidations. We are led to the view, therefore, that the uniform failure of the kidney in these rats is due to a lack of unsaturated fatty acids essential to normal kidney tissues.

The results with moderately high and low protein diets indicate that high protein diets aid in the breakdown of the kidney. The most recent workers on this subject have come to the conclusion that exceedingly high protein diets cause the kidneys to hypertrophy but do not produce any recognizable nephritis (2–4). It would then seem probable that on a complete diet high protein intake will not break down the rat kidney in a period of a year but

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if the diet is subnormal in other respects (low in unsaturated fatty acids) high protein intake may hasten the degeneration.

The authors are aware that great care should be exercised in drawing conclusions concerning human nutrition from results with rats. It seems worth while to point out, however, that the human diet is often exceedingly low in fats of any kind and that when fats are added they usually contain little of the acids more unsaturated than oleic. Butter and coconut oil are the chief table fats and beef fat is probably equally poor as a source of unsaturated acids. It is possible that our high carbohydrate and protein diets, carrying very little of the unsaturated oils, are contributing factors to poor health. The addition of egg yolk and cod liver oil to diets may often improve the patient because of the fatty acid rather than the vitamin content. For example, cures of anemia with cod liver oil have been reported and it has been shown that there is a relation between experimental anemia and the unsaturated fatty acids of the blood plasma (44, 45). The prevalence of dry skins and abnormal kidneys may be directly attributable to improper fat intake. The nerve tissue, kidneys and other organs contain several unsaturated acids. If the liver is limited in its ability to produce these acids, they should be plentifully supplied through the diet.

CONCLUSIONS.

1. Fat-free diets regularly produce kidney lesions in the rat, which have been observed in every case at autopsy. Kidney degeneration probably causes the death of the rat in most cases.

2. High protein diet seems to increase the severity of the kidney degeneration so that bloody urine appears more frequently.

3. The small emaciated animals on fat-free diets drink twice as much water as their controls and eat the same amount of food. The excess water is not lost through the urine.

4. Ovulation often is irregular or ceases entirely in fat-free animals. When a curative oil is fed, ovulation is resumed within a few days.

5. Vitamin E is not a controlling factor in the disease resulting from fat-free diet, but may affect the size of the males somewhat.

6. Female rats on the fat-free diet will mate when ovulation

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occurs and will produce litters. The very poor litters are attributed to the general poor condition of the mother.

7. With few exceptions, males on a fat-free diet will not mate, while their controls, receiving 10 drops of lard, mate and sire normal litters. Those males fed fat-free diets, which do mate, cannot sire litters.

8. In this new type of sterility the normal sex responses are lost, while in the sterility resulting from lack of vitamin E the sex response is retained after the loss of the seminiferous epithelium.

9. Rats suffering from the low fat disease are not cured by the saturated fatty acids (stearic, palmitic, myristic, lauric, or lower acids).

10. These same rats are cured by linoleic acid (either isolated in pure state, or in olive oil, lard, corn oil, poppy-seed oil, linseed oil, or egg lecithin).

11. The phospholipid, egg lecithin, is of no more value than pure methyl linolate.

12. Oleic acid in butter and coconut oil is of doubtful value but certainly it is not equal to linoleic acid.

13. Complex, unsaturated oils (like corn oil, linseed oil, cod liver oil) appear to be more effective curative substances than a single fatty acid or phospholipid.

14. High grade butter fat (3 per cent of the total diet) does not cure the skin condition, supporting the view that vitamin A and vitamin E deficiency is not involved.

15. The hypothesis is put forward that warm blooded animals in general cannot synthesize appreciable quantities of linoleic acid. The synthesis of other unsaturated acids, including linolenic, is probably equally limited.

16. Linoleic acid (and possibly other acids) therefore is an essential fatty acid.

The authors wish to express their thanks to Professor C. M. Jackson for his active cooperation in this work.

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A STUDY OF GLUTATHIONE.

II. THE DETERMINATION OF REDUCED GLUTATHIONE IN TISSUES.

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The iodine titration proposed by Tunnicliffe (10) has been employed by most workers for the estimation of reduced glutathione in tissues. On account of its importance this method will be discussed in detail, but first it is desired to mention two methods which have appeared recently.

Flatow (3) has described a method in which an excess of ferricyanide is used to oxidize the glutathione in a strongly alkaline solution. The excess ferricyanide is titrated with indigo sulfonic acid. The method as described is applied to blood, in which case it is necessary to make corrections for uric acid and t'ioneine.

The other method, proposed by Gabbe (6), at The Thirteenth International Physiological Congress, but not yet published in full, is also based on the use of ferricyanide. The glutathione is treated with an excess of ferricyanide in hydrochloric acid solution. The excess ferricyanide is then allowed to react with potassium iodide and the free iodine is titrated with thiosulfate. It will be shown that reduced glutathione is not oxidized by ferricyanide in a solution which is more than 0.01 N in hydrochloric acid (Table III). Consequently, oxidation of the glutathione does not occur until the iodine is liberated. Essentially, therefore, this method is an iodine titration in which iodine is added in excess and the excess is back-titrated with thiosulfate. The method appears to have no advantage over the usual titration with iodine.

Thompson and Voegtlin (9) adopted the iodine titration after some scrutiny. They preferred to continue the titration until the nitroprusside reaction disappeared rather than to use starch as an

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internal indicator. On the other hand, Blanchetière and Melon (2) maintained that the nitroprusside reaction is not sufficiently sensitive to indicate the presence of a small quantity of glutathione. They used starch to show the end-point. Perlzweig and Delrue (8) recommended the use of starch provided there is sufficient iodide present to stabilize the starch-iodine color. Turner (11) reported a comparison of starch and nitroprusside as indicators. The use of nitroprusside led to figures which were 5 to 15 per cent

TABLE I.

*Titration of Glutathione with Iodine.**

Glutathione used.	Initial volume.	KI added.	GSH found with starch indicator.		GSH found with nitroprusside indicator.
mg.	*cc.*	*gm.*	*mg.*	*per cent*	*per cent*
10.0	50	0.00	10.8	108	
10.0	50	0.01	10.5	105	
10.0	50	0.05	10.4	104	
10.0	50	0.10	9.9	99	
10.0	50	0.50	9.8	98	
61.4	50	0.50	58.7	96	
61.4	200	2.0	60.2	98	
12.3	50	0.5	12.1	99	
12.3	50	0.5	12.3	100	91
12.3	50	0.5	12.3	100	94
5.0	100	1.0	5.2	104	88
5.0	100	1.0	5.2	104	92

* A recrystallized sample of the tripeptide was used. By titration with potassium ferricyanide and by colorimetric comparison with cysteine it was found to contain 97 per cent of GSH.

The iodine used was approximately 0.01 N except for the sixth and seventh titrations when it was twice this strength.

lower than those obtained with starch. Potassium iodide was not added.

The question as to the validity of the results obtained with the iodine titration is of significance since this method has been widely used. Inasmuch as Okuda (7) found that the iodine uptake of cysteine varies with the concentration of acid and with the temperature, one might expect the same to be true of glutathione. An examination of the method as applied to crystalline glutathione has been made with due consideration of the various points mentioned. Table I shows the results.

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Most striking is the effect of iodide on the consumption of iodine. This effect is to be attributed to the elimination of side reactions rather than to stabilization of the starch-iodine color. An experiment was designed to test this interpretation. An excess of iodine was allowed to act in the absence of iodide and then the excess was titrated in the presence of potassium iodide and starch. 30 per cent more iodine was taken up by the glutathione than when potassium iodide was added before the excess of iodine. Since in both instances there was sufficient iodide to stabilize the starch-iodine color, it is evident that the extra consumption of iodine is in no way concerned with this color. The effect of the iodide is on the course of the reaction between iodine and glutathione.

The contention of Blanchetière and Melon and of Turner that the use of the nitroprusside reaction to indicate the end-point leads to low results, has been confirmed. The differences between the figures obtained by the two methods for the determination of this end-point agree well with Turner's observations. However, the sensitivity of this reaction is so dependent on the procedure that it may possibly give better results in the hands of other workers. Starch is obviously the indicator of choice.

The effect of a decrease in the concentration of GSH is to increase the uptake of iodine although the effect is not marked. A dilution of 5 times brought about an increase of about 4 per cent; a dilution of 12 times, an increase of 7 per cent.

In contrast to cysteine, variations in the acidity from 0.05 to 3.0 cc. of 5 N hydrochloric acid in 50 cc. changed the titer little more than the experimental error. These figures are not included in Table I.

It is thus shown that in the absence of other tissue extractives reduced glutathione can be estimated to within a few per cent by titration with iodine in the presence of potassium iodide and with starch as an internal indicator. However, the presence of other tissue constituents may invalidate a method which is excellent for the pure substance. Although Tunnicliffe found that urea, uric acid, creatinine, glucose, and fructose are not attacked by iodine, there are present in some tissues substances other than glutathione which do react with iodine and therefore interfere with the method. The iodine titration of tissue extracts will be considered further in comparison with a new method for the determination of reduced glutathione.

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The new method is based on the oxidation of GSH within a narrow range of pH with potassium ferricyanide. Oxidation of sulfhydryl to disulfide occurs with the formation of an equivalent amount of ferrocyanide. The ferrocyanide is estimated colorimetrically by conversion into Prussian blue. Certain details of the method were suggested by Folin's ferricyanide method for blood sugar (4) and two of his reagents have been adopted.

As the acidity increases the oxidation potential of ferricyanide increases, but the reducing intensity of GSH decreases at such a rate that at pH values below 5.7 it is not completely oxidized by ferricyanide. Some tissue constituents act similarly but they cease to reduce ferricyanide at an acidity which is not sufficient to interfere with the complete oxidation of GSH. Accordingly, it is possible to select for the oxidation a pH at which glutathione is completely oxidized while other substances are not attacked or only partially so. A buffered solution of pH 5.9 was found to be satisfactory.

An important feature of the method is the blank in which formaldehyde prevents the oxidation of glutathione without interfering with the oxidation of a number of other substances. Although this action of formaldehyde is not entirely specific for the sulfhydryl group, it affords a valuable correction for the reducing power of other substances which react with potassium ferricyanide.

Reagents.

Potassium Ferricyanide.—A 0.01 N solution of potassium ferricyanide, purified by the method of Folin (4), is used.

Buffer.—This is Sörensen's phosphate buffer. The stock solutions are M/7.5 primary potassium phosphate and M/7.5 secondary sodium phosphate. For use 1 volume of the secondary phosphate is mixed with 9 volumes of the primary phosphate. The pH of the buffer is close to 5.9.

Stock Solution of Cysteine.—Dissolve 157.5 mg. of cysteine hydrochloride in a little water containing 10 cc. of 5 N hydrochloric acid. Dilute to 100 cc. This solution is 0.01 N in cysteine. It will not lose an appreciable amount of reducing power in 6 to 8 hours if kept in the ice box, but it must be renewed or standardized daily. It should always be standardized even when freshly prepared.

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Standard Cysteine Solution.—Dilute 10 cc. of the stock solution to 50 cc. to make a solution 0.002 N in cysteine. This solution will keep for 3 or 4 hours at room temperature. 1 cc. is equivalent to 0.614 mg. of reduced glutathione.

Ferric Sulfate Reagent.—The reagent described by Folin is used with one change. The amount of gum arabic is doubled. No difficulty has been experienced in keeping the reagent 4 to 6 weeks.

Formaldehyde.—The U. S. P. 40 per cent solution is used.

Standardization of Cysteine.

All of the samples of cysteine hydrochloride which have been prepared have been found to be slightly impure. It is therefore necessary to determine the cysteine content of each sample. One method for this is the titration of a standard solution of iodine in alcohol with an alcohol solution of the cysteine hydrochloride.

A more satisfactory method is the titration with potassium ferricyanide at a pH of about 7.4. Measure 25 cc. of the stock solution of cysteine into an Erlenmeyer flask and add 10 cc. of phosphate buffer of pH 7.4 (a mixture of 200 cc. of the primary with 800 cc. of the secondary phosphate stock solutions). Now add sufficient sodium hydroxide to neutralize the hydrochloric acid present and titrate immediately with 0.01 N potassium ferricyanide until a permanent yellow remains. The end-point is determined by comparison with a blank which contains a volume of water equal to the volume of the titrated solution at the end of the titration and 0.2 cc. of the ferricyanide. This amount of ferricyanide is then subtracted from the titer. It is easily possible to duplicate titrations to within 0.1 cc.

Procedure.

To make clear certain steps in the procedure, two facts must be mentioned: (1) Cysteine and glutathione undergo autooxidation very slowly in acid solution but when the pH is brought to 5.9 appreciable loss of these substances may occur in a few minutes; and (2) it is important that the volumes of standard and unknown should be approximately equal at the time the Prussian blue is developed. When these facts are taken into account, the procedure which follows has been found convenient in most cases.

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Measure into a 100 cc. flask a volume of tissue extract, prepared by the method of Folin and Wu (5), which contains between 1 and 2 mg. of glutathione. Titrate another portion with standard alkali. Congo red is used as the indicator. Dilute the sample to such a volume that when the alkali and buffer have been added the total volume will be 25 cc. Now add 5 cc. of buffer and, without delay, the requisite amount of alkali, and then the potassium ferricyanide which should be added a few drops at a time. The flask is shaken and not more than 3 or 4 drops of ferricyanide are added in excess

TABLE II.

*Relation between the Amount of Glutathione Used and the Color Produced.**

Standard, cc. 0.002 N cysteine.	Glutathione used.	Reading.	GSH found.	
	mg.	*mm.*	*mg.*	*per cent*
0.50	0.20	31.9	0.192	96
0.50	0.25	25.1	0.245	98
0.50	0.30	21.3	0.288	96
0.50	0.40	15.7	0.391	97
0.50	0.50	12.6	0.487	97
2.00	0.50	54.0	0.456	91
2.00	1.00	25.6	0.96	96
2.00	1.20	21.5	1.14	95
2.00	1.40	18.0	1.37	98
2.00	1.60	16.0	1.54	96
2.00	1.80	13.9	1.77	98
2.00	2.00	12.8	1.92	96
2.00	2.50	9.8	2.51	100
2.00	3.00	8.3	2.96	99

* The standard was set at 20 mm.

so that the solution has a distinctly yellow color. Wait 15 minutes. Because the reaction is slow an apparent excess of ferricyanide may disappear after a few minutes and necessitate the addition of more. Now add 3 cc. of the ferric sulfate reagent and allow 10 minutes for the color to develop. Dilute to volume and compare in the colorimeter with the standard prepared from cysteine.

If there is a large difference in the intensity of the colors of standard and unknown, the comparison in the colorimeter is difficult. The color of the excess ferricyanide appears more intense in

the solution containing the less Prussian blue when the blue colors are matched. Folin has shown that this difficulty can be overcome by the addition of ferricyanide to the solution containing the more Prussian blue. For further details, reference should be made to Folin's paper.

The standard is prepared as just described. The amount of cysteine is so chosen that the unknown reads between 15 and 30 with the standard set at 20 mm. The color of the excess ferricyanide is more likely to interfere if there is too great a difference between standard and unknown. Table II shows the limitations in this respect.

If a solution contains less than 1 mg. of glutathione in 15 cc., smaller flasks may be used. Less than 0.15 mg. of glutathione cannot be determined accurately for the color is too faint to be read in the colorimeter. With these small quantities the volume in which the color is developed should be kept as small as possible.

The volume of tissue extract for the blank may be doubled to advantage since the blanks are usually small. Place the sample in a 25 cc. flask, add 0.5 cc. of formaldehyde, and proceed as before. One precaution must be observed. After the addition of the ferric sulfate the blanks are immediately placed in the dark since the ferric ion sensitizes them to light. This does not cause difficulty in reading them in the colorimeter as the action is slow. An exposure of 15 minutes to the diffuse light of the laboratory may cause a definite color in what would otherwise be a colorless blank.

It is best to provide a series of standards which contain 0.1, 0.2, 0.3, and 0.5 cc. of the standard cysteine solution for comparison with the blanks. A blank which has less color than would match a 0.25 cc. standard cannot be read accurately. The blank is then so low that it is sufficient to compare it with the appropriate standards with the unaided eye.

The procedure as outlined can be varied if the precautions noted are observed. A few words should be added concerning the volume in which the Prussian blue is formed. A small difference between the volumes of standard and unknown is not of consequence but a larger difference (25 cc.) may cause considerable error. Development in a large volume with subsequent dilution yields less color in 100 cc. than development in a small volume with subsequent dilution. This is due to a latent period in the

development of color in the larger volume since the colors will be the same after several hours. The color is inversely proportional to the volume when diluted after full development.

Large amounts of salts tend to retard the development of color. The quantity of salts encountered in tungstic acid filtrates is negligible.

If oxalates are present the solution becomes sensitive to light after the addition of the iron, but no difficulty is experienced when the solution is shielded from the light and read in the colorimeter without delay.

TABLE III.

*Influence of pH on Determination of Reduced Glutathione.**

pH	GSH in crystalline preparation, mg. GSH found in 2.0 mg. sample.	GSH in liver extract.		
		Total reducing power as GSH.	Blank as GSH.	GSH found.
		mg.	*mg.*	*mg.*
2.0	0.00			
5.2	1.10			
5.5	1.70	0.83	0.06	0.77
5.7	1.91	0.91	0.07	0.84
5.9	1.95	0.94	0.08	0.86
6.2		1.01	0.13	0.88
6.7		1.04	0.13	0.91
7.4	1.95	1.05	0.13	0.94

* The extract was a tungstic acid filtrate of fresh rabbit liver. 5 cc. were used for each determination.

The straight line relation between the intensity of color and amount of glutathione is shown in Table II. The same relation holds for cysteine. Deviations from the straight line relation occur only when there is a wide divergence from the standard.

DISCUSSION.

Although the blank is relied on to eliminate the effect of some of the substances other than glutathione, the action of formaldehyde is not specific. Thus, the oxidation of uric acid at pH 7.4 is partially inhibited by formaldehyde. Doubtless there are other substances which are affected similarly. In consideration of this point a number of compounds was examined as to their possible interference with the method.

1. Cysteine.—This behaves in every way like glutathione.

2. Thioneine.—The oxidation of thioneine is completely inhibited by formaldehyde. However, at pH 5.9 it gives only one-tenth as much color as an equal weight of glutathione. About 8 mg. of thioneine is the average of the amounts found in human blood by Benedict and Newton (1). Such a quantity would increase the apparent glutathione values obtained by this method by 0.8 mg., a negligible amount. It is of course possible to determine the amount of thioneine and apply the proper correction.

3. Uric Acid.—This is not oxidized at pH 5.9.

4. Phenols.—Quinol, pyrogallol, and epinephrine are readily oxidized by potassium ferricyanide. Their oxidation is not affected by formaldehyde.

5. Hexuronic Acid of the Suprarenal Cortex.—This compound, obtained from Szent-Györgyi, is rapidly oxidized at pH 5.9 and its oxidation proceeds equally well in the presence of formaldehyde.

6. Glucose, Lactic Acid, and Urea.—These are not attacked. The values obtained by this method are maximal. All errors except those of manipulation are positive. If any other substances are oxidized, the apparent amount of glutathione will be increased unless this oxidation occurs to the same extent in the blank or unless some of the tissue extractives have an influence on the oxidation of the GSH. The oxidation of GSH is not affected by other tissue constituents since GSH added to tissue extracts can be quantitatively recovered. To a blood extract which contained 0.26 mg. of GSH were added 1.15 mg. of GSH. Found 1.37 mg. 0.77 mg. of GSH was added to a liver extract which contained 2.38 mg. Found 3.13 mg.

The influence of pH on the oxidation of GSH and on the values of the total reducing power and the blank of tissue extracts is well illustrated by Table III. It will be noted that a variation of 0.1 pH unit either side of 5.9 is of no consequence. The amount of buffer used can easily maintain the pH within this range when the solution has previously been made just alkaline to Congo red. The pH of 5.9 allows complete oxidation of glutathione with a minimum of oxidation of other substances.

Comparison of the amounts of glutathione found in yeast, liver, and kidney extracts by the iodine titration and by the new method (Table IV) reveals some striking differences. The iodine titrations

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indicate the presence of 20 to 100 per cent more glutathione than the new method. Although most of the titrations were performed before the influence of iodide was appreciated, consideration of the

TABLE IV.

*Comparison of Iodine Titrations with Colorimetric Estimations.**

Tissue.	Colorimetric estimation.					Iodine titration.	
	Weight of tissue.	Total reducing power of GSH.	Blank of GSH.	GSH found.	GSH in 100 gm. tissue.	GSH in 100 gm. tissue.	Indicator.
	gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Yeast.	1.33	1.69	0.06	1.63	123	227	Nitroprusside.
	1.60	3.23	0.56	2.67	167	216	"
	1.25	2.04	0.19	1.85	148	174	"
	2.10	2.79	0.08	2.71	129	156	"
	1.20	2.18	0.21	1.97	164	204	"
Blood (hog).	1.50	0.34	0.00	0.34	23	42	Nitroprusside.
	1.50	0.31	0.00	0.31	21		"
Blood (rabbit).	1.50	0.43	0.00	0.43	29	26	Nitroprusside.
						33	Starch.
Liver (hog).	1.00	2.12	0.66	1.46	146	327	Nitroprusside.
	1.00	3.12	0.65	2.47	247	410	"
Liver (rabbit).	1.00	1.33	0.49	0.84	84	154	Nitroprusside.
	1.00	3.06	0.63	2.43	243	319	"
						368	Starch.
Kidney (rabbit).	1.00	0.74	0.19	0.55	55	61	Nitroprusside.
						88	Starch.
Muscle (rabbit).	1.50	0.63	0.00	0.63	42	30	Nitroprusside.
						46	Starch.

* Potassium iodide was added to those extracts which were titrated with iodine in the presence of starch. Potassium iodide was not added to the other extracts.

data of Table I shows that the omission of iodide cannot account for the large differences found. When applied to extracts of blood and muscle the results of the two methods are not far apart. The iodine titration, however, tends to give a high result when the

indicator is starch, whereas the result is lower when the disappearance of the nitroprusside reaction is taken as the end-point. This is in full accord with the results of other workers. Further reference to Table I shows that the lack of sensitivity of the nitroprusside reaction is sufficient to account for the low values. It is apparent that the iodine titration cannot be used for the estimation of glutathione in extracts of yeast, liver, and kidneys. Its use with extracts of other tissues should be thoroughly investigated before reliance is placed on the results.

It is perhaps significant that when the iodometric and colorimetric values are close together the blank is very small or zero; when the two values are divergent, the blank is large. It appears that some reducing factors in the tissues which react with iodine are eliminated to a large extent in the ferricyanide oxidation by the use of the low pH and the blank. These reducing factors are particularly abundant in yeast, liver, and kidneys.

Thioneine as a constituent of blood is of particular interest since, like glutathione, it has reducing power associated with a sulfhydryl group. Accordingly the iodine titration of thioneine alone and in the presence of GSH was studied. It was found that a sample of thioneine obtained from T. B. Johnson used 0.9 as much iodine as an equal weight of glutathione. This consumption of iodine is 9 times the number of equivalents of ferricyanide reduced by thioneine. It might very well account for the difference in the results obtained by the two methods with blood filtrates. It seems likely that the errors involved in the iodine titration of blood filtrates are those involved in the titration of GSH itself plus the error due to thioneine.

Aside from its greater specificity the new method has a further advantage over the iodine titration in that much smaller amounts of tissue are required. When small concentrations of glutathione are involved, it is more precise since glutathione uses more than an equivalent amount of iodine at low concentrations (Table I).

SUMMARY.

In the course of a study of the iodine titration of reduced glutathione it was found that iodide is necessary to make the reaction proceed in a straightforward manner. Starch is much to be preferred to nitroprusside as the indicator.

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A new method for the estimation of reduced glutathione in tissues is described.

The new method is compared with the iodine titration method. The values found for glutathione in extracts of yeast, liver, and kidneys by titration with iodine are much larger than those found by the new method. Since the latter gives maximal values, the iodine titration is considered as not trustworthy for these tissues. With blood and muscle there is fair agreement between the two methods. Any disagreement in the case of blood is probably due to thioneine.

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THE BASAL METABOLISM OF YOUNG COLLEGE WOMEN IN FLORIDA.

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Several years ago preliminary observations on the basal metabolism of a group of normal students at the Florida State College for Women gave indication that the metabolism was lower than that predicted with the Harris-Benedict and Aub-Du Bois standards. As a result of these observations this study was begun. Data have been collected on 52 subjects between the ages of 17 and 25 years.

EXPERIMENTAL.

Subjects.—Young college women, who were either native Floridans or had lived in the state most of their lives and whose physical records showed them to be normal individuals, were selected as subjects. For the most part they were students who had taken courses in which they had studied energy metabolism; thus they were acquainted with the apparatus and the conditions necessary for an accurate determination. There was a fine spirit of cooperation and at no time was there evidence of any marked psychic disturbances which might influence the results.

The subject came to the laboratory between 7.00 and 7.30 a.m. before she had eaten breakfast. The determination was made between 12 and 13 hours after the last meal. Following a rest period of $\frac{1}{2}$ to $\frac{3}{4}$ of an hour, the metabolism was measured during two successive periods of about 8 to 10 minutes each. Before each test the mouth temperature was taken in order to rule out any possible condition of fever. The metabolism determination was not made during the menstrual period.

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Apparatus.—The apparatus used was a Sanborn-Benedict. Tracings of the respirations were recorded on a kymograph, and a time clock gave the time indications. In addition, readings were made on the scale and two stop-watches were used. The apparatus was kept in very good condition and frequently tested for leaks. During each determination a test was made for a leak by placing two 20 gm. weights on the spirometer for the last 4 or 5 minutes and noticing the general slope of the tracing. In the very few when the tracing showed any signs of a leak the experiment was discarded. Unabsorbed carbon dioxide was tested for by passing a sample of the residual gas after an observation through barium hydroxide. To make the data more reliable an alcohol check should have been made. I regret to say that none was made. However, in the case of two of the subjects the metabolism was measured on a student form of respiration apparatus and the results checked very closely with those observed on the Sanborn-Benedict apparatus.

RESULTS AND DISCUSSION.

The results of the metabolism determinations are given in Table I. They represent the average of all the observations made on each subject. For thirty-one of the 52 subjects the results represent the average of observations made on 2 successive days. In the case of the remaining twenty-one they represent the average of observations made on 3 to 8 days scattered over a period of several months. In making up the average only those observations which agreed within 10 cc. were used.

The average metabolism of the group of 52 subjects is 1236 calories per 24 hours or 33.4 calories per square meter per hour. This is a deviation from the prediction of −9.9 per cent (Harris-Benedict) and −10.6 per cent (Aub-Du Bois).

If it is accepted that the standards for women should be lowered by about 5 per cent, the average metabolism of this group still is lower than the normal limits. By the Harris and Benedict prediction there are only two whose metabolism is above the predicted and three by the Aub and Du Bois prediction. All the others are below. It is interesting to note that by the Harris and Benedict prediction thirty, or 58 per cent, of the group are below −10 per cent; twenty-eight, or 54 per cent, are below by the Aub and

TABLE I.

Average Basal Metabolism of Normal Young Women.

Subject No.	Age.		Weight.	Height.	Pulse.	O_2 consumed per min.	Total heat production per 24 hrs. (Benedict).	Heat production per sq. m. per hr. (Du Bois).	Deviation of actual from predicted.	
									Harris-Benedict prediction.	Aub-Du Bois prediction.
	yrs.	*mos.*	*kg.*	*cm.*		*cc.*	*cal.*	*cal.*	*per cent*	*per cent*
1	17	2	43.2	157	63	155	1077	32.5	−15.9	−18.7
2	17	4	51.1	163	59	169	1174	32.3	−13.8	−19.3
3	17	7	58.9	152	59	178	1237	33.4	−12.7	−12.1
4	18	0	61.3	159	69	170	1181	30.5	−18.0	−19.7
5	18	0	52.7	168	74	211	1466	38.4	−5.7	+1.1
6	18	1	47.5	156	65	159	1105	31.9	−15.9	−16.0
7	18	2	47.3	152	75	167	1160	34.5	−11.1	−9.2
8	18	3	58.9	158	67	194	1348	35.3	−5.5	−7.1
9	18	10	63.4	170	74	208	1445	35.0	−2.8	−7.9
10	19	0	54.5	159	58	162	1126	30.2	−18.5	−20.5
11	19	2	56.2	153	72	182	1265	34.9	−8.9	−8.1
12	19	2	54.3	159	70	187	1299	35.1	−5.9	−7.6
13	19	2	56.1	162	81	184	1278	33.7	−8.9	−11.3
14	19	3	54.2	156	71	169	1174	33.4	−14.5	−12.3
15	19	4	49.6	164	66	162	1126	31.0	−16.2	−18.4
16	19	7	45.6	152	69	165	1146	33.2	−10.5	−10.3
17	19	7	49.9	169	68	194	1348	36.2	−0.2	−2.1
18	19	8	52.2	165	65	171	1188	31.9	−13.4	−13.8
19	19	8	55.7	157	73	188	1306	35.1	−5.7	−5.1
20	19	9	55.3	154	63	174	1209	33.3	−11.8	−10.0
21	19	10	55.6	160	75	171	1188	31.7	−14.5	−14.3
22	19	11	58.9	163	88	209	1452	37.3	+1.8	+0.8
23	20	0	52.2	147	77	170	1181	34.4	−11.3	−7.0
24	20	0	54.6	169	66	186	1292	33.4	−7.4	−9.7
25	20	1	52.8	162	76	168	1292	34.9	−5.4	−5.7
26	20	1	51.6	163	67	171	1188	31.1	−12.5	−16.0
27	20	2	52.9	161	67	166	1153	30.5	−15.5	−17.5
28	20	4	56.3	165	61	197	1369	35.6	−2.7	−3.8
29	20	5	47.0	150	68	151	1049	31.4	−11.7	−15.1
30	20	6	55.9	163	50	171	1188	31.1	−14.7	−15.9
31	20	6	72.8	153	57	197	1369	33.5	−12.2	−9.4
32	20	7	48.8	163	63	162	1126	31.0	−7.7	−16.2
33	20	7	51.1	158	68	176	1223	34.2	−11.8	−7.6
34	20	9	43.7	157	66	159	1105	32.8	−12.6	−11.4
35	20	10	60.3	163	67	192	1334	34.1	−7.0	−7.8
36	20	11	47.0	156	76	175	1216	31.2	−6.1	−15.8

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TABLE I—*Concluded.*

Subject No.	Age.		Weight.	Height.	Pulse.	O_2 consumed per min.	Total heat production per 24 hrs. (Benedict).	Heat production per sq. m. per hr. (Du Bois).	Deviation of actual from predicted.	
									Harris-Benedict prediction.	Aub-Du Bois prediction.
	yrs.	*mos.*	*kg.*	*cm.*		*cc.*	*cal.*	*cal.*	*per cent*	*per cent*
37	21	0	70.1	160	86	211	1466	35.5	−3.7	−4.1
38	21	0	56.7	160	77	176	1223	32.2	−12.9	−12.9
39	21	0	50.0	153	63	184	1278	36.7	−3.0	−0.8
40	21	4	44.6	153	71	163	1133	34.2	−10.9	−7.6
41	21	5	59.6	157	83	177	1230	32.2	−13.3	−13.0
42	21	6	58.6	166	53	173	1202	30.5	−15.3	−17.6
43	21	6	66.8	164	63	189	1313	32.0	−12.1	−13.5
44	21	7	62.8	175	63	193	1341	32.5	−9.4	−12.1
45	21	10	55.9	160	65	177	1230	32.8	−11.0	−11.3
46	21	10	53.6	160	60	178	1237	33.4	−9.1	−9.7
47	22	1	43.2	153	73	158	1098	33.8	−12.0	−8.7
48	22	7	56.0	162	61	172	1195	31.5	−13.5	−14.9
49	23	4	41.9	153	80	172	1195	36.9	−2.7	−0.3
50	24	0	55.5	161	52	178	1237	30.9	−12.1	−16.8
51	24	6	41.7	153	66	176	1223	38.0	+0.3	+2.7
52	25	1	50.2	163	70	185	1285	35.5	−2.6	−4.1
Average.....			54.5	159	68	178	1236	33.4	−9.9	−10.6

Du Bois prediction. All the very low results were repeated several times so that we feel quite confident that the figures represent basal values. It would thus seem that there is some evidence that the basal metabolism of southern college women tends to be lower than that predicted for young women of the same age and living in the north. The actually measured metabolism of a group of twenty Wellesley College students reported by Gustafson and Benedict (1) showed a deviation of only −1.8 per cent from the predicted metabolism. However, in a study made in New Orleans, Hafkesbring and Borgstrom (2) observed that the average metabolism of a group of nine subjects, two of which were women, was 16 per cent below the Harris-Benedict standards and 18 per cent below the Aub-Du Bois standards. Also Hafkesbring and Collett (3) found that their average basal metabolism was 5 to 8 per cent below the

Harris-Benedict standard. Hindmarsh (4) reported in a study of 76 white students (twenty-six of whom were women) living in Sydney, New South Wales, Australia, that the basal metabolic rate of the women showed on the average a reduction of 10.5 per cent from the Aub-DuBois predicted value. With the Harris-Benedict predicted value the average decrease in metabolic rate was 8.6 per cent. These results compare very closely with the figures obtained here in Florida. However, we do not have as wide a range of variation. Our results range from −18.5 per cent to +1.8 per cent (Harris-Benedict) and −20.5 per cent to +2.7 per cent (Aub-Du Bois), while Hindmarsh reported a range of −31.3 per cent to −10.2 per cent (Harris-Benedict) and −33.8 per cent to −11.7 per cent (Aub-Du Bois).

TABLE II.

Variation in the Oxygen Consumption.

Results are expressed in cc. per minute.

Subject No.	Jan.	Feb.	Mar.	Apr.	May.	June.	July.	Aug.	Oct.	Nov.	Dec.
24			191	184	185						185
26	175		171		171						169
32		169				162	158			163	167
33		176	172							179	
35	190	197	195	187	194					191	
45	182		171	182	177				178	179	
48						170	172	174			
50			187	180		168					

Eight of the subjects were observed for a period extending over 3 to 6 months. The results of these observations are tabulated in Table II. Although there are not enough data to draw any definite conclusions it appears that there has been no change in the metabolism with a change in season. In this section of Florida the temperature may range from 5–10° during January and February to 25–30° during the summer months. It is interesting to note that in New Orleans where the climate is quite similar to that in northern Florida, Hafkesbring and Collett (3) found their metabolism to be 5 per cent higher in cold weather (2–10°) than in hot (20–27°). Gustafson and Benedict (1) report that the study of the metabolism of the Wellesley College students strongly

suggests that the metabolism tends to be at a low level in winter and to rise to a higher level during the spring and summer. Further studies are in progress to determine the effect of seasonal variation on metabolism.

Eight of the subjects were majors in the department of Physical Education (Subjects 8, 14, 15, 22, 24, 28, 29, 30), one was a student assistant (Subject 35), and one was an instructor (Subject 50). The percentage variation in the metabolism for these subjects is brought together in Table III. The average percentage is −9.0 per cent (Harris-Benedict) and −10.6 per cent

TABLE III.

Percentage Variation in Metabolism for Students Majoring in Physical Education.

Subject No.	Harris-Benedict prediction.	Aub-Du Bois prediction.
	per cent	*per cent*
8	−5.5	−7.1
14	−14.5	−12.3
15	−16.2	−18.4
22	−1.8	−0.8
24	−7.4	−9.7
28	−2.7	−3.8
29	−11.7	−15.1
30	−14.7	−15.9
35	−7.0	−7.8
50	−12.1	−16.8
Average.....	−9.0	−10 6

(Aub-Du Bois). This agrees closely with the average for the entire group. This is rather interesting since it was thought that perhaps the increased physical activity on the part of these students might have a tendency to raise their metabolism. de Almeida (5) found that one of his subjects living in Brazil was able within a year to increase his metabolism from 22.7 calories per square meter per hour to 32 calories by leading a life of greater physical activity.

SUMMARY.

1. Figures are presented for the basal metabolism of 52 subjects at Florida State College for Women varying in age from 17 to 25 years.

2. The average metabolism of the group is −9.9 per cent (Harris-Benedict standards) and −10.6 per cent (Aub-Du Bois standards).

3. With a few data it appears that there is no change in metabolism with change in season.

4. Eight of the subjects who indulged in greater physical activity had an average metabolism agreeing closely with the average of the entire group.

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VARIATIONS IN INORGANIC CONSTITUENTS OF THE PANCREATIC JUICE DURING CONSTANT DRAINAGE OF THE PANCREATIC DUCTS.

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Most of the data concerning the inorganic constituents of the pancreatic juice have been obtained from experiments on animals with open fistulæ, the juice being contaminated with other body fluids or exposed to the air. Many of the data are also the result of acute experiments which lasted only a short time, where the rate of flow of juice was changed by stimuli of various sorts. It appeared desirable to study pancreatic juice obtained from unanesthetized animals with as little stimulus to the flow of juice as possible.

The method devised by Rous and McMaster (1) for the collection of bile under sterile conditions, and adapted for use in collecting pancreatic juice by Elman and McCaughan (2), seemed best suited for our purpose. This method consists of intubating the pancreatic duct with a small cannula to which is attached a long piece of rubber tubing which leads from the cannula to the peritoneal cavity for a distance of about 18 inches before emerging to the outside, where it is attached to a collecting receptacle. By this method, the tubing is covered within a day or two by adhesions from the omentum and affords no ingress for organisms from the outside to the pancreas. The pancreatic juice can, in this way, be collected without contact with air or contaminating material and the pancreas is protected from postoperative infection.

All the material from which the data in this report were obtained was collected from dogs by the method described above. Instead of using rubber bags as recommended by Elman and McCaughan

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(2) we collected the fluid in sterile glass bottles, under oil. In this way juice was collected with a minimum loss of CO_2. Each bottle was fitted with a 2-hole stopper carrying a long glass inlet tube extending to the bottom of the bottle and a short exit tube plugged with cotton. This modification apparently did not affect the secretion of the juice but it is conceivable that the rate of flow may have been impeded by the hydrostatic pressure which developed as the bottle filled.

No difficulty was encountered with the operative technique necessary for this sort of experimentation. The dogs were anesthetized by an intraperitoneal injection of sodium amytal, the pancreas carefully dissected away from the duodenal wall, and all the ducts tied or cannulated. The ducts which were not used for cannulation were doubly tied, turned back upon themselves, and sewed into the tissue about the pancreas at a distance from the duodenum. In order to make doubly sure that the severed duct did not grow into the duodenum, a piece of omentum was drawn between these structures. All animals were studied after the experiment to make sure that there were no intact ducts leading into the duodenum. The animals were kept in clean cages and the bottles containing the secreted pancreatic juice replaced by others morning and night, or oftener, when necessary.

Vomiting, so often reported as a disturbing factor in experiments on animals with permanent pancreatic fistulæ, did not occur during the course of these experiments. It was found in previous experiments that animals with pancreatic fistulæ became very thirsty when deprived of water for a short time. When given water, they drank large quantities rapidly and vomiting ensued, which was very difficult to control. We, therefore, were careful to keep a generous supply of fresh water before the animals at all times.

Before the animals recovered from the anesthetic, which usually required from 12 to 36 hours, they were given 50 to 200 cc. of tap water by stomach tube every few hours. We feel that vomiting is not directly caused by the presence of the cannula in the pancreatic duct, but that dehydration and thirst with the subsequent intake of large quantities of water into the stomach are the chief cause. That the vomiting present after complete drainage of the pancreatic juice is due to gastric irritability as suggested

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by Hartmann and Elman (3) is a possibility. Hartmann and Elman state that the animals of their series which received no treatment lived but 5 to 8 days. Some of our animals lived for longer periods, Dog 14 living 23 days after institution of the drainage, with no treatment other than careful feeding and watering. Our animals had moderate appetites throughout these experiments, and we had no difficulty getting them to eat the small quantities of food given them at short intervals. No attempt was made to control the intake of the various substances for which the pancreatic juice was analyzed, the ordinary animal house fare, a general mixed diet, being given.

Analytical Methods.

Chloride was determined by the Van Slyke method as modified by Wilson and Ball (4), total carbon dioxide by the method of Van Slyke and Stadie (5), and inorganic phosphates by the method of Fiske and Subbarow (6). Sodium and potassium were determined on ashed samples of juice, sodium by the method of Kramer and Gittleman (7), and potassium by the method of Kramer and Tisdall (8), the modifications introduced by Kerr (9) being used for both methods. Calcium was determined by the method of Clark and Collip (10). For the determination of magnesium Briggs' (11) method, with slight modifications described by Ball (12), was used. Total nitrogen was determined by the method of Folin and Denis (13). Values for specific gravity were obtained by weighing a measured portion of the juice and dividing by the weight of an equal amount of water at the same temperature. Total solids were determined by drying a measured portion at 105–110° overnight and weighing the residue. This residue was then carefully ashed in a platinum crucible by the Stolte (14) method and reweighed to determine the total ash. Hydrogen ion concentration was determined by means of the hydrogen electrode, with the refill technique described by Cullen (15).

Results.

The data in this report were obtained from five dogs with permanent pancreatic fistulæ. For comparison with the data which we have obtained, the composition of pancreatic juice as

reported by some earlier investigators is given in Table I. These data, in general, are in fairly close agreement with ours.

Chlorides determined on samples of juice collected from five dogs on the day following institution of the drainage showed variations in concentration from 88 to 138 mM (Tables II to VI). There were also marked variations in chloride concentrations of juices obtained from the same dog on different days. In all the animals there was a tendency for the chloride concentration to decrease as the drainage progressed. An illustration of this

TABLE I.

Average Composition of Pancreatic Juice As Reported by Other Investigators (Recalculated in Terms of mM per Liter Where Necessary).

Investigator.	Gm. per 100 cc.			mM per liter.						
	Sp. gr.	Total solids.	Ash.	Na	K	Ca	Mg	Cl	HCO_3	PO_4
Schmidt (1854) (16).	1.006	1.93	0.680	149	12.5	0.7	0.5	55		0.6
Schumm (1902) (17).	1.010	1.39	0.852	144	6.4			51		
Frouin and Gerard (1912) (18).				161	11.7	0.3	0.1	49		0.02
Wohlgemuth (1912) (19).	1.006	1.30		132	2.5	Trace.	Trace.	129		2.3
Gamble and McIver (1928) (20).				148	7.0	3.0		81	79	0.5
Hartmann and Elman (3).				144*				50	59	0.13

* Total base instead of sodium.

decrease may be found in the data obtained from Dog 14 (Table II), the chloride concentration falling, in later samples, well below one-half of the concentration found in earlier samples. The calcium concentration of the juice also decreased as the drainage progressed, but there was no close relationship between the calcium and chloride concentration.

The total inorganic cation and anion concentrations in the various samples are approximately equivalent (Tables V and VI) as pointed out by Gamble and McIver (20). This is in line with the low protein content which is demonstrated by the nitrogen

TABLE II.

Dog 14. Weight 22 Kilos; Operation April 17, 1928.

Day after operation.	Hour at end of collection.	Amount of pancreatic juice.	pH	Cl	Ca	Cl excreted per day.	Base excreted per day.*
		cc.		mM per l.	mM per l.	mM	mM
1	9 a.m.	60		88	2.2		
	5 p.m.	70†		62	2.2		
2	9 a.m.	140		68	2.5		
	5 p.m.	60		76		14.08	30.00
3	9 a.m.	50		71			
	5 p.m.	45		81		7.20	14.25
4	9 a.m.	12†		100			
	5 p.m.	Lost.					
5	9 a.m.	"					
	5 p.m.	70					
6	9 a.m.	120†	7.16	105	2.3		
	5 p.m.	60		100		18.60	27.00
7	9 a.m.	170	7.56	96	2.1		
	5 p.m.	Lost.					
8	9 a.m.	210		88			
	5 p.m.	Lost.					
9	9 a.m.	120†		86	2.0		
	10 "	20		95			
	5 p.m.	20†		92		14.06†	24.00†
10	9 a.m.	125		76			
	5 p.m.	140		58		17.60	39.75
11	9 a.m.	140		93			
	5 p.m.	40		95		17.82	27.00
12	9 a.m.	180		78			
	5 p.m.	30					
13	9 a.m.	240†	7.36	72			
	5 p.m.	30†		69		19.35†	40.50†
14	9 a.m.	Lost.					
	1 p.m.	120†	8.04	61			
	5 "	50		66			
15	9 a.m.	100	7.77	64	1.1		
	3 p.m.	60		53			
	5 "	30		62		11.44	28.50
16	9 a.m.	240†	7.80	58	0.8		
	5 p.m.	150		64		23.52†	58.50†
17	9 a.m.	150		55	0.9		
	5 p.m.	50		56		11.05	30.00

* Calculated from the volume of juice excreted per day, 150 mM of base per liter being assumed.

† Indicates loss of portion of sample.

TABLE II—*Concluded.*

Day after operation.	Hour at end of collection.	Amount of pancreatic juice.	pH	Cl	Ca	Cl excreted per day.	Base excreted per day.*
		cc.		*mM per l.*	*mM per l.*	*mM*	*mM*
18	9 a.m.	Lost.					
	5 p.m.	150		50			
19	9 a.m.	120†		55	0.9		
	5 p.m.	Lost.					
20	10 a.m.	240†	7.30	54			
	1.30 p.m.	80	7.90	44			
	5 p.m.	120		45		21.88†	66.00†
21	9 a.m.	240†	7.61	39			
	12 noon.	100		41			
	5 p.m.	50		47		15.81†	58.50†
22‡	9 a.m.	180†		46			
	p.m.	10		56			

‡ May 9. In the morning the animal appeared very weak. At 4.30 p.m. it was found dead in the cage. Weight, 15.8 kilos.

TABLE III.

Dog 7. Weight 15.0 Kilos; Operation January 11, 1928.

Day after operation.	Hour at end of collection.	Amount of pancreatic juice.	Cl	Ca
		cc.	*mM per l.*	*mM per l.*
1	9 a.m.	120*		
	5 p.m.	60	108	1.4
2	9 a.m.	100		
	5 p.m.	45	116	1.5
3	9 a.m.	100	111	1.6
	5 p.m.	Lost.		
4	9 a.m.	70*	106	1.8
	5 p.m.	Lost.		
5	9 a.m.	45*		
	5 p.m.	40	89	1.1
6	9 a.m.	Lost.		
	5 p.m.	60*	91	1.5
7	9 a.m.	50*	85	1.2
	5 p.m.	Lost.		
8	9 a.m.	120*	92	1.3
	5 p.m.	40*	93	1.2
9†	9 a.m.	60*	82	1.3

* Indicates loss of portion of sample.

† On January 20 the flow of juice stopped. The dog lived for 8 days in apparent good health, when it pulled the tube out and died.

and total solid determinations. In calculating total anion concentrations we have assumed that all the carbon dioxide is present as bicarbonate because data were not available for calculating free and combined CO_2. The errors involved in such calculations are too slight to affect our discussion as Ball (12) has shown that pancreatic juice contains about 1 mM of free CO_2.

Several determinations of hydrogen ion concentration were

TABLE IV.

Dog 11. Weight 20 Kilos; Operation February 29, 1928.

Day after operation.	Hour at end of collection.	Amount of pancreatic juice.	Cl	Ca
		cc.	*mM per l.*	*mM per l.*
1	9 a.m.	20	125	
2	9 "	75	124	1.6
	5 p.m.	55		
3	9 a.m.	140*	96	1.4
	5 p.m.	80	82	
4	9 a.m.	140	70	2.1
	5 p.m.	Lost.		
5	9 a.m.	65	94	1.9
6	9 "	140	93	2.0
	5 p.m.	120*	96	
7	9 a.m.	220	89	1.8
	5 p.m.	120	87	1.8
8	9 a.m.	240*	86	1.8
	5 p.m.	120*	66	
9	9 a.m.	240*	66	1.5
	5 p.m.	120*	71	1.6
10	9 a.m.	180	56	1.5
	5 p.m.	80	82	
11	9 a.m.	15	93	2.4

* Indicates loss of portion of sample.

carried out on juices from Dog 14 (Table II). It is not assumed that these data represent an accurate estimate of the pH of the pancreatic juice, as some carbon dioxide was probably lost from the juice during collection and consequently the figures may be too alkaline. They are given as approximate figures and indicate that the pH of pancreatic juice may vary considerably.

Of the inorganic constituents in solution in pancreatic juice, sodium, chloride, and bicarbonate predominate; calcium, potas-

TABLE V.

Dog 9. Weight 13 Kilos; Operation December 6, 1928.

Day after operation.	Hour at end of collection.	Amount of pancreatic juice.	Sp. gr.	Gm. per 100 cc.			mM per liter.								
				Total solids.	Ash.	N	Na	K	Ca	Mg	Cl	CO_2	PO_4	Total cations.	Total anions.
		cc.													
1	5.00 p.m.	26	1.019	6.392	0.915	0.870	145	4.6	2.5	0.5	138	15	0.6	153	154
2	9.00 a.m.	65	1.010	3.307	0.968	0.382	155	4.6	1.7	0.5	109	47	0.4	162	156
	5.30 p.m.	23	1.007	2.564	0.917		155	5.1			94	60	0.4	160+	154
3	10.30 a.m.	72	1.007	2.249	0.890	0.230	149	4.4	1.8	0.5	107	43	1.2	156	151
	7.00 p.m.	38	1.007	2.325	0.890	0.238	144	4.8			102	45	0.9	149+	148
4	9.00 a.m.	40	1.007	2.193	0.901	0.275	142	4.6	1.6	0.7	96	46	0.8	149	143
	5.30 p.m.	2										16			
5	9.00 a.m.	15	1.004	3.024	0.836		136	4.7			120	18	1.1	141+	139
	5.00 p.m.	10									121	24	1.4		146
6	9.00 a.m.	22	1.007	2.404	0.874	0.276	142	5.0			118	23	1.3	147+	142
	5.00 p.m.	Lost.													
7	9.00 a.m.	68	1.005	1.840	0.874	0.210	142	5.2	1.6	0.7	107	37	1.1	150	145

TABLE VI.

Dog 8. Weight 17.2 Kilos; Operation November 30, 1928.

Day after operation.	Hour at end of collection.	Amount of pancreatic juice.	Sp. gr.	Gm. per 100 cc.			mM per liter.								
				Total solids.	Ash.	N	Na	K	Ca	Mg	Cl	CO_2	PO_4	Total cations.	Total anions.
		cc.													
1	9.00 a.m.	28	1.007	2.358	0.906	0.210	144	3.7	1.8	0.3	122	29	0.2	150	151
	5.00 p.m.	40	1.005	1.446	0.902	0.085	147	4.1	1.4	0.3	110	43	0.1	153	153
2	10.00 a.m.	62	1.006	1.396	0.872	0.079	142	3.7	1.2	0.3	101	50	0.2	147	151
	6.30 p.m.	5		1.740	0.880	0.092		3.7			103	39			142
3	9.00 a.m.	Lost.													
	5.00 p.m.	"													
4	9.00 a.m.	10		1.610	0.900	0.149		4.4			94	87	0.7		182

sium, magnesium, and phosphate are present in much smaller quantities. During drainage the total base shows very little tendency to decrease. There is relatively little change in any of the cations except calcium, the concentrations of sodium, potassium, and magnesium remaining relatively constant. Chloride and carbon dioxide concentrations vary considerably, but our data confirm Gamble and McIver's observation that the decrease in chloride ion is associated with an increase in bicarbonate. The decrease in concentration of chloride in the juice is probably associated with a diminished chloride concentration in the blood found by Gamble and McIver (21).

Our data, as well as those of Gamble and McIver (20), indicate that the concentration of total base in pancreatic juice is fairly constant at approximately 150 mM per liter. Assuming this concentration for all the samples of juice obtained from Dog 14 (Table II), we have calculated the approximate loss of base by excretion through the pancreas. The loss of chloride is calculated from the data obtained in that experiment. As drainage progressed, the loss of chloride increased somewhat and then decreased markedly. The total amount of chloride and base excreted by the animal daily varied between wide limits, as the rate of flow fluctuated markedly. The average hourly secretion during the night period, when the animal was resting, was about the same as during the day period, when the animal was active and taking food. This does not necessarily mean that the intake of food did not stimulate the flow of juice. The animals were usually fed at the time the collecting receptacle was removed, so that the effect of food would be found in the night as well as in the day sample.

SUMMARY.

A study of pancreatic juice excreted by five dogs with permanent pancreatic fistulæ reveals a marked variation in the concentrations of chloride, bicarbonate, and calcium. The concentrations of chloride and calcium decreased as drainage progressed. With the fall in chloride the bicarbonate concentration in the juice rose, the total anion concentration remaining nearly constant. The total cation concentration closely paralleled the anion concentration. The hydrogen ion concentration of the pancreatic juice varied between pH 7.16 to 8.04.

We wish to express our thanks to Dr. D. W. Wilson and Dr. I. S. Ravdin for valuable suggestions and help, and to Dr. J. H. Jones for carrying out some of the calcium determinations.

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A METHOD FOR THE PREPARATION OF BLOOD FILTRATES FOR THE DETERMINATION OF SUGAR.

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I. Effect of Zinc Salts upon Reducing Non-Sugars.

A few years ago the writer observed that tungstate filtrates of blood yield considerably higher reducing non-sugar values (non-fermentable reducing substances) when determined with an alkaline ferricyanide reagent than when copper is used as the oxidizing agent. The average reduction values, expressed in equivalents of glucose, were 21 mg. per cent with the modified Shaffer-Hartmann method and 40 mg. per cent with the ferricyanide reagent. As a consequence, the latter gave an average of 20 mg. per cent higher apparent sugar (total reduction) values than the Shaffer-Hartmann reagent (1). In seeming contradiction to this we encounter in the literature reports that the Hagedorn-Jensen method, with a ferricyanide reagent, yields results not higher than those obtained by several copper reduction methods (2, 3).

It would be entirely misleading, however, to compare copper reduction methods with the Hagedorn-Jensen procedure without taking into consideration that while in the former the sugar is generally determined in tungstate filtrates, the filtrates involved in the Hagedorn-Jensen method are prepared by a vastly different procedure, consisting in heat coagulation of the proteins in the presence of zinc hydroxide. With the amount of non-sugar reducing substances in such filtrates unknown, an adequate correlation of the sugar values is obviously impossible.

Experiments recorded in Table I furnish information as to the quantity of reducing non-sugars in zinc hydroxide filtrates,[1]

[1] The Hagedorn-Jensen method, in which 0.1 cc. of blood is used in 1:120 dilution, is unsuited for the detection of differences of moderate magnitude.

determined by both a copper and a ferricyanide reagent. Table I also includes the reducing non-sugar values in the tungstate filtrates of the same blood specimens. Comparison of Column 1 with Column 2 and of Column 3 with Column 4 shows, in accordance with our previous findings, that in any single filtrate ferricyanide produces much higher reducing non-sugar values than does copper.[2] This may be due to a higher oxidation intensity of the ferric ion or to a distinct selectiveness of the cupric ion

TABLE I.

Reducing Non-Sugars in Folin-Wu and Hagedorn-Jensen Filtrates.

Experiment No.	Folin-Wu filtrates.		Hagedorn-Jensen filtrates.	
	Copper reagent.* (1)	Ferricyanide reagent.† (2)	Copper reagent. (3)	Ferricyanide reagent. (4)
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	15	38		24
2	19	44		24
3	12	32		26
4	14	34	5	15
5	24	49	7	24
6	20	43	12	27
7	15	36	8	20
8	18	37	8	18
9	18	42	7	21
10	17	37	10	23
11	26	49	10	31

* A modification of the Shaffer-Hartmann reagent.

† Original Hagedorn-Jensen reagent.

For the present experiments it was transposed, so to speak, to a macro scale by increasing the concentration of the reagents 10-fold, but otherwise retaining the original proportions. This enabled us to prepare 1:10 filtrates in the following manner: In a 50 cc. volumetric flask 25 cc. of 4.5 per cent zinc sulfate solution are precipitated with 5 cc. of N sodium hydroxide. To the suspension of zinc hydroxide are admixed 5 cc. of blood, and the mixture heated in a boiling water bath to complete coagulation, cooled, and diluted with water to 50 cc. Dry paper is used for filtration. It was ascertained that this technique yields slightly lower reducing non-sugar values than the original Hagedorn-Jensen method.

[2] Herbert and Groen recently made similar observations when employing several analytical procedures for the examination of blood filtrates prepared by three different methods of deproteinization (4).

in the oxidation of the non-fermentable reducing substances of blood, or possibly to both factors.

The information of signal interest, however, is that the Hagedorn-Jensen filtrates actually do contain considerably smaller amounts of reducing non-sugars than the Folin-Wu filtrates. It so happens that they give with ferricyanide reducing non-sugar values on the whole not higher than those obtained with copper reagents in Folin-Wu filtrates (Columns 1 and 4), a coincidence that dissipates the apparent contradiction pointed out above.

The remarkably low values of reducing non-sugars, as determined in Hagedorn-Jensen filtrates by the copper reagent (Column 3), prompted an inquiry into the possibility of establishing conditions under which zinc would completely precipitate in a single operation not only the proteins but also the non-sugar reducing substances. And if such conditions could be found, as a second essential prerequisite we stipulated rapidity and simplicity of technique, attributes that render the tungstate method of Folin and Wu so exceedingly convenient. Our study resulted in the development of a procedure which, we believe, fairly satisfies both of our stipulations.

II. Methods of Precipitation by Zinc.

In the Hagedorn-Jensen procedure, mixing blood with freshly precipitated zinc hydroxide results in but a partial precipitation of the proteins; to carry the process to completion, heating of the mixture is indispensable. If, however, a zinc salt is added to laked blood, and thereafter the reaction is rendered approximately neutral or slightly alkaline, the precipitation of proteins is accomplished in the cold without any further operation, as in analogous procedures with mercury salts. Moreover, adequate regulation of the hydrogen ion concentration effects at the same time substantially the complete removal of the non-sugar substances which reduce alkaline copper solutions, so that the protein-free blood filtrates yield with copper reduction methods true sugar values.

On the basis of this observation we devised a procedure for the preparation of blood filtrates which was outlined in a brief report a year ago (5). We shall describe here two variations of our method,

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each having some special advantages under different experimental conditions but otherwise giving the same results.

Macro Technique.

Reagents for First Procedure.

Solution I.—10 per cent solution of $ZnSO_4 \cdot 7H_2O$.

Solution II.—0.5 N NaOH.

The two solutions must be so related that when 10 cc. of Solution I are titrated with Solution II, 10.8 to 11.2 cc. are required to produce a permanent pink color with phenolphthalein. The 10 cc. of Solution I are diluted with 50 to 70 cc. of water before titration; it is essential to add the sodium hydroxide slowly with continuous shaking.

Procedure.—To prepare blood filtrates in the conventional 1:10 dilution, lake 1 volume of blood in 7 volumes of water, introduce 1 volume of Solution I, mix; then add with continuous shaking 1 volume of Solution II, stopper the flask, shake well, and filter through dry paper after a few minutes. For accurate work measure the blood with an Ostwald pipette calibrated "to contain" and rinse with the laking water.

Reagents for Second Procedure.

Solution I.—12.5 gm. of $ZnSO_4 \cdot 7H_2O$ are dissolved in water, 125 cc. of 0.25 N H_2SO_4 are added, and the mixture diluted with water to 1 liter.

Solution II.—0.75 N NaOH.

The two solutions must be so related that when 50 cc. of Solution I are titrated with Solution II, 6.70 to 6.80 cc. are required to produce a permanent pink color with phenolphthalein. The titration must be carried out by slow addition of the sodium hydroxide with continuous shaking.

Procedure.—For the preparation of 1:10 blood filtrates, lake 1 volume of blood in 8 volumes of Solution I, add 1 volume of Solution II, stopper the flask, shake vigorously, and filter through dry paper after a few minutes.

The advantages of this variation over the first are elimination of one measurement, more rapid filtration, and a larger yield of filtrate. The advantage of the first procedure is greater elasticity

when the method is to be applied to different protein concentrations, as in the case of corpuscles or plasma.

To prepare 1:10 filtrates of corpuscles, lake 1 volume of corpuscles in 5 volumes of water (use a pipette "to contain" and rinse with laking water); precipitate with 2 volumes of 10 per cent zinc sulfate and 2 volumes of 0.5 N sodium hydroxide.

To precipitate the proteins of blood plasma or serum at 1:10 dilution, introduce 1 volume of plasma into 8 volumes of water and use 0.5 volume of each of the precipitants.

Micro Technique.

Both variations of the method are applicable to sugar determinations in 0.1 or 0.2 cc. of blood.

Reagents for First Procedure.

Solution I.—1.8 per cent $ZnSO_4 \cdot 7H_2O$.

Solution II.—0.1 N NaOH.

The two solutions must be so related that when 10 cc. of Solution I, diluted with 60 cc. of water, are slowly titrated with Solution II, 12 to 12.2 cc. are required to produce a permanent pink color with phenolphthalein.

Procedure.—Introduce into a test-tube or 25 cc. Erlenmeyer flask 5.8 cc. of water, measure accurately 0.2 cc. of blood in a reliable capillary pipette, and lake in the water. Rinse the pipette several times. Add 1 cc. of Solution I, mix, then add with continuous shaking 1 cc. of Solution II. Stopper the flask or test-tube, shake well, and filter through dry paper after a few minutes. A little over 5 cc. of filtrate—representing 1:40 dilution of the blood—are readily obtained, provided the filter paper employed is not too thick (70 mm. size of Schleicher and Schüll No. 597 was found adequate for this work). The sugar is determined by the modified Shaffer-Hartmann method in 5 cc. of filtrate, corresponding to 0.125 cc. of blood. To determine sugar in 0.1 cc. of blood, use 5.9 cc. of water for laking; in this case 5 cc. of the filtrate correspond to 0.0625 cc. of blood.

Reagents for Second Procedure.

Solution I.—To 100 cc. of Solution I, prepared for the second macro procedure, add 325 cc. of water.

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Solution II.—0.15 N NaOH.

Procedure.—Introduce into a test-tube or 25 cc. Erlenmeyer flask 6.8 cc. of Solution I, measure accurately 0.2 cc. of blood, and deliver into the solution with continuous gentle shaking. Rinse the pipette several times. Take care that the small clumps of blood are thoroughly disintegrated and extracted. (Without this precaution some occluded sugar might escape determination and the results would be too low. Smearing of blood on the sides of

TABLE II.

Reducing Non-Sugars Determined by Shaffer-Hartmann Method in Filtrates Prepared from Human Blood by Three Methods of Deproteinization.

Experiment No.	Method of deproteinization.		
	Folin-Wu.	Hagedorn-Jensen.	Somogyi.
	Reducing non-sugars per 100 cc. blood, in terms of glucose.		
	mg.	*mg.*	*mg.*
1	14	5	0
2	24	7	0
3	20	12	Trace.*
4	15	8	0
5	18	8	Trace.
6	18	7	0
7	19	8	0
8	19	8	4
9	17	10	Trace.
10	26	10	0

* "Trace" denotes reduction values corresponding to 0.01 to 0.03 cc. of 0.005 N iodine, quantities sometimes occurring in washings of yeast however carefully washed beforehand.

the test-tube has the same effect.) Next add 1 cc. of Solution II, stopper, shake well, and filter through dry paper after a few minutes. Filtration is very rapid in this procedure.

III. Determination of Sugar in Zinc Filtrates.

The water-clear filtrate obtained by either of the procedures described is ready for sugar determination without further preparation, provided a buffered copper solution, like the modified Shaffer-Hartmann reagent, is used. Otherwise neutralization of

the filtrate must precede the addition of the copper reagent, as Folin recommends in his own method (6).

In Tables II to IV to follow are presented analytical data characteristic of the reduction values of zinc filtrates. Table II

TABLE III.

Showing that Sugar Values in Zinc Filtrates of Human Blood Are the Same As True Sugar Values Obtained by Fermentation Technique.

Experiment No.	Tungstate filtrates.			Zinc filtrates.
	Apparent sugar.	Reducing non-sugars.	True sugar.	
	Per 100 cc. blood, in terms of glucose.			
	mg.	*mg.*	*mg.*	*mg.*
1	93	16	77	73
2	233	16	217	219
3	140	17	123	122
4	218	19	199	199
5	117	19	98	99
6	108	23	85	86
7	109	22	87	89
8	103	21	82	80
9	255	23	232	234
10	103	21	82	82
11	131	19	112	112
12	79	19	60	59
13	52	15	37	36
14	156	24	132	131
15	134	24	110	110
16	93	17	76	74
17	141	15	126	126
18	148	20	128	133
19	121	15	106	105
20	107	17	90	90
21	124	16	108	107
22	98	16	82	79
23	110	15	95	88
24	84	13	69	69
25	100	13	87	84

contains examples to show, without necessitating commentary, that the new procedure yields filtrates which, after the sugar is removed by yeast, contain virtually none of the reducing substances in blood.

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In Table III is given a series of twenty-five sugar determinations with human blood, carried out by the Shaffer-Hartmann method simultaneously on tungstate filtrates and zinc filtrates. The true sugar values in tungstate filtrates are represented by the difference between apparent sugar (total reduction) values and reducing non-sugars[3] (non-fermentable reducing substances, "residual reduction"). As can be seen, the sugar values in zinc filtrates are identical with the true sugar values.

TABLE IV.

Showing That Reduction Values in Zinc Filtrates of Human Blood Represent True Sugar.

Experiment No.	Reduction values, in terms of glucose, per 100 cc. of:				Glucose added to sugar-free blood.
	Sugar-free blood.		Blood after addition of glucose.		
	Tungstate filtrates.	Zinc filtrates.	Tungstate filtrates.	Zinc filtrates.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
1	15	0	216	201	200
2	18	Trace.*	121	98	100
3	18	0	225	201	200
4	12	Trace.	209	196	200
5	12	"	215	199	200
6	24	5	426	405	400
7	16	0	266	248	250
8	14	0	218	200	200
9	18	Trace.	412	401	400
10	19	"	523	505	500
11	17	"	170	150	150
12	26	0	179	151	150

*"Trace" denotes reduction values corresponding to 0.01 to 0.03 cc. of 0.005 N iodine, quantities sometimes occurring in washings of yeast however carefully washed beforehand.

A more direct evidence is adduced from the experiments recorded in Table IV. These were executed as follows: Blood was laked in 3 volumes of water and about 10 per cent of purified yeast suspended in it for 10 to 15 minutes at room temperature. The

[3] The reducing non-sugar values in Table III are on the whole somewhat lower than reported in a previous paper (7). This discrepancy is due to the fact that the copper reagent now in use has a lower iodide content than the one previously used.

suspension was then centrifuged, the fermented blood poured off, and subjected to a second sharp centrifugation in order to insure the complete removal of yeast. The sugar-free blood was divided in two portions, to one of which a known amount of glucose was added. Each sample was then deproteinized by both the tungstate and the zinc procedure and the reduction of the filtrates determined by the Shaffer-Hartmann method. The results show that added glucose is quantitatively recovered in the zinc filtrates. Nothing is lost in the process of precipitation, and nothing added from the non-sugar reducing substances. The tungstate filtrates —it is again demonstrated—give reduction values representing with fair approximation the sum of added glucose and the non-fermentable reducing substances (first and last columns).

If zinc filtrates contain sugar as the sole substance oxidizable by alkaline copper reagents, then different methods for the determination of sugar should yield identical results. In a previous study (8) we found this to be the case. Parallel sugar determinations were performed by the Folin-Wu method, by Benedict's method, by Folin's modification of the Folin-Wu method, and by the Shaffer-Hartmann method, with the result that the four procedures give substantially identical sugar values in zinc filtrates.

SUMMARY.

A rapid, simple, and inexpensive procedure for the precipitation of blood proteins is presented. In the same operation the non-fermentable reducing substances are precipitated also, so that the protein-free filtrates give true sugar values with alkaline copper solutions.

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THE RATE OF DISAPPEARANCE OF FOREIGN SUGAR FROM THE BLOOD STREAM.

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The estimation of the concentration of foreign sugars in the blood in the presence of glucose is a problem of great importance in both physiology and pathology. Somogyi (1) observed that yeast removed glucose quantitatively from dilute solutions and that this action is limited to the fermentable sugars. Although Somogyi interpreted this as a process of adsorption, Benedict (2) proved definitely this action is caused by the fermentation of the sugar. Corley (3–5), Raymond and Blanco (6), and Blanco (7), have used this method to determine glucose in the presence of foreign sugars and for the estimation of sugars other than glucose injected into animals. We have injected galactose and xylose into rabbits (Figs. 1 and 2) in an attempt to find the mathematical expression for the rate of disappearance of the foreign sugar from the blood stream.

Let us assume that the rate of disappearance of the sugar is proportional to the concentration, C, of the sugar itself, which is decreasing.

$$\frac{dC}{dt} = -\alpha C \tag{1}$$

Integrating, we get the equation of a monomolecular reaction.

$$C = Ae^{-\alpha t} \tag{2}$$

where α will be a constant dependent upon the unit of time employed and A will be dependent upon the amount injected into the blood and the particular method used for the estimation of the sugar.

In order to find the value of α, we have to eliminate A, and for

this purpose we can most conveniently take the values of C at two different times, C_1 at times t_1 and C_2 at times t_2.

$$C_1 = Ae^{-\alpha t_1} \tag{3}$$

$$C_2 = Ae^{-\alpha t_2} \tag{4}$$

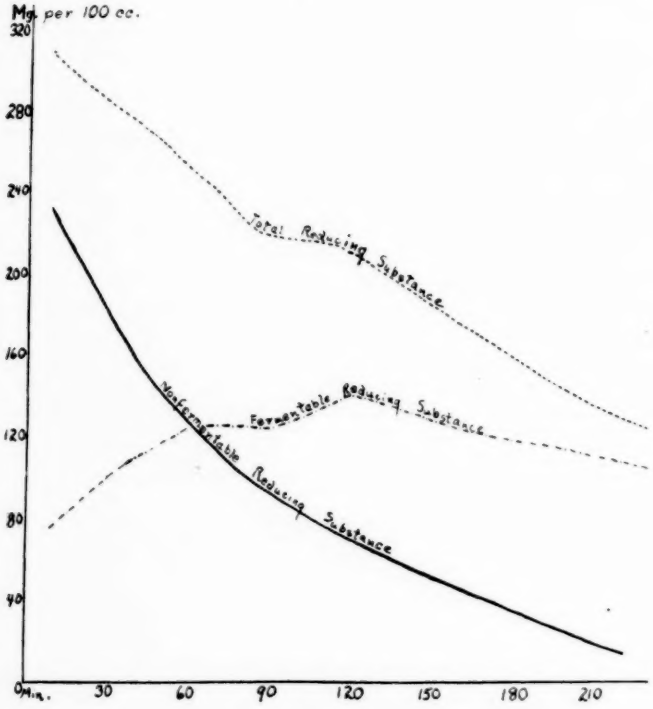

FIG. 1. Concentration of total, fermentable, and non-fermentable reducing substances in the blood following the injection of 2.5 gm. of xylose into the marginal ear vein of the rabbit.

Let us assume that the concentration, C, is twice C_2; then dividing Equation 3 by Equation 4 we get

$$\frac{C_1}{C_2} = 2 = \frac{e^{-\alpha t_1}}{e^{-\alpha t_2}} = e^{\alpha (t_2 - t_1)} \tag{5}$$

$$\alpha\,(t_2 - t_1) = \log_e 2 = 2.3 \times \log_{10} 2 \tag{6}$$

$$\alpha = \frac{0.694}{t_2 - t_1} = \frac{0.694}{70 \pm 5} = 1 \times 10^{-2} \tag{7}$$

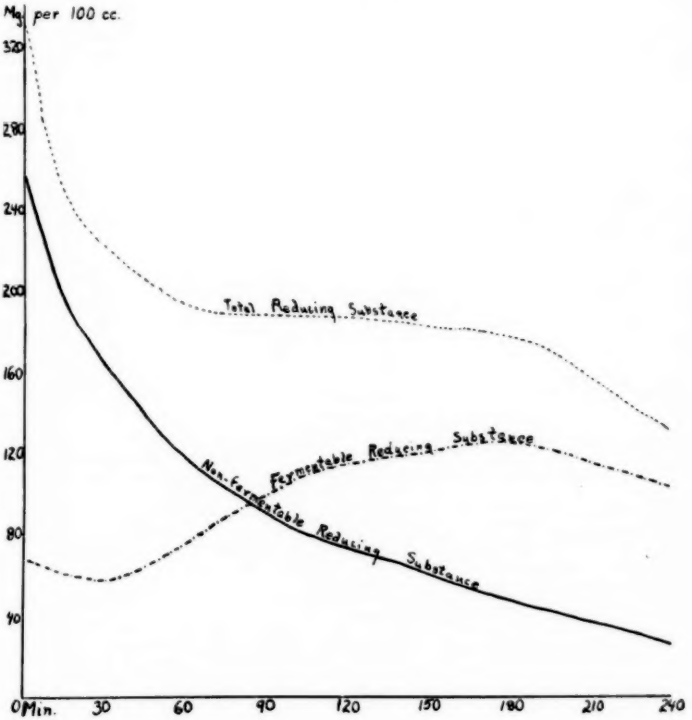

FIG. 2. Concentration of total, fermentable, and non-fermentable reducing substances in the blood following the injection of 3 gm. of galactose into the marginal ear vein of the rabbit.

Taking any two values of C_1 and C_2 so that one is twice the other, we find that $t_1 - t_2$ is equal to a little over an hour, hence we can calculate the value of α as 1×10^{-2}. Substituting this value in the formula for our observed values of C, we find A constant within the limits of our experimental accuracy as seen from Tables I and

II and the results of Corley (3–5) (Tables III and IV) and Blanco (7) (Tables V and VI).

The similarity of the equation $C = Ae^{-\alpha t}$ to that of the law governing the velocity of a monomolecular chemical reaction is at

TABLE I.

Effect of Injection of Xylose into Marginal Ear Vein of Rabbit.

Time.	Total reducing substance.	Non-fermentable reducing substance.	Fermentable reducing substance.	$e^{-\alpha t}$ $\alpha = 1 \times 10^{-2}$	A
a.m.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*		
10.15	124	28	96		
10.35	2.5 gm. xylose injected.				
10.45	343	244	99	0.905	270
11.25	300	138	162	0.607	230
12.00	251	107	144	0.427	258
p.m.					
12.30	232	73	169	0.316	230
1.20	213	46	167	0.192	238
2.15	135	24	114		

TABLE II.

Effect of Injection of 5 Gm. of Galactose into Marginal Ear Vein of Rabbit.

Time.	Total reducing substance.	Non-fermentable reducing substance.	Fermentable reducing substance.	$e^{-\alpha t}$ $\alpha = 1 \times 10^{-2}$	A
a.m.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*		
9.40	121	32	89		
9.45	5 gm. galactose injected.				
9.50	440	323	107	0.951	340
10.15	343	230	113	0.741	310
10.45	331	170	161	0.549	310
11.15	251	124	127	0.407	302
12.00	190	96	94	0.301	312
p.m.					
1.00	181	52	129	0.165	318
1.45	162	34	128	0.105	310

once apparent. However, there is one striking difference. The physical meaning of A in a chemical reaction is the initial concentration of the reacting substance; when $t = 0$ then $C_0 = A$. In ordinary chemical reactions C_0 is the easiest quantity to deter-

mine; but the initial concentration of a substance injected into the blood stream of a living animal cannot be measured because of the time required for the withdrawal of the blood. Hence: instead of solving for α in terms of a known A, C, and t, we have calculated α by elimination of A with any two values of C_1 and C_2, and, assuming constancy of α throughout, have solved for A.

TABLE III.

Effect of Injection of 1 Gm. of Xylose into Marginal Ear Vein of Rabbit (Data Taken from Corley (3)).

Time.	Non-fermentable sugar.	$e^{-\alpha t}$ $\alpha = 10^{-2}$	A
min.	*mg. per 100 cc.*		
5	110	0.951	115.7
65	56	0.522	107.5
125	35	0.287	122.0
245	11	0.091	121.5

TABLE IV.

Effect of Injection of Galactose into Marginal Ear Vein of Rabbit (Data Taken from Corley (4)).

Time.	3 gm. galactose.			2 gm. galactose.			2 gm. galactose.		
	Non-fermentable sugar.	$e^{-\alpha t}$ $\alpha = 10^{-2}$	A	Non-fermentable sugar.	$e^{-\alpha t}$ $\alpha = 10^{-2}$	A	Non-fermentable sugar.	$e^{-\alpha t}$ $\alpha = 10^{-2}$	A
min.	*mg. per 100 cc.*			*mg. per 100 cc.*			*mg. per 100 cc.*		
0	281	1.000	280	226	1.000	220	263	1.000	260
60	139	0.549	260	101	0.549	190	131	0.549	240
120	84	0.301	270	49	0.301	170	69	0.301	230
180	36	0.165	230	30	0.165	190	33	0.165	210

The constancy of A is alike proof of the constancy of α and the form of the equation used.

Method.

0.2 cc. of blood was dropped into 2.3 cc. of water and then 1 cc. of a 33 per cent suspension of yeast, prepared by centrifugation according to Benedict (2), was added. This yeast gave a negligible reducing blank. We then added 0.5 cc. of equal parts of sodium tungstate and $\frac{2}{3}$ N sulfuric acid. After centrifuging, 2 cc.

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of the supernatant fluid were used for a sugar estimation by the method of Hagedorn and Jensen (8).

SUMMARY.

The monomolecular reaction velocity equation is found to apply to the rate of disappearance of foreign sugars injected into the blood stream of rabbits. It is thus possible to determine the concentration of the non-fermentable reducing substance at any time in the blood when its concentration at any definite time is known, since the logarithm of the concentration of the foreign sugar at any time is proportional to the time after injection.

TABLE V.

Effect of Injection of 2 Gm. of Xylose into Marginal Ear Vein of Rabbit (Data Taken from Blanco (7)).

Time.	Non-fermentable sugar.	$e^{-\alpha t}$ $\alpha = 1 \times 10^{-3}$	A
min.	*mg. per 100 cc.*		
15	380	0.861	440
30	280	0.741	390
45	250	0.638	400
105	190	0.350	520

TABLE VI.

Effect of Injection of 2 Gm. of Galactose into Marginal Ear Vein of Rabbit (Data Taken from Blanco (7)).

Time.	Non-fermentable sugar.	$e^{-\alpha t}$ $\alpha = 1 \times 10^{-3}$	A
min.	*mg. per 100 cc.*		
15	480	0.861	555
30	390	0.741	520
60	280	0.549	510

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MICRO COLORIMETRIC STUDIES.

II. ESTIMATION OF PHOSPHORUS: MOLYBDIC ACID-STANNOUS CHLORIDE REAGENT.

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(Received for publication, January 31, 1930.)

Although other methods for the estimation of phosphorus have appeared since the publication of the paper of Kuttner and Cohen (1), we found that method to possess certain advantages which we wish to point out in this communication. We shall also comment upon the use of the reagents as well as certain precautions for their preparation, not previously published. This will help to obviate difficulties which may be encountered by those using the method.

We are in agreement with the authors that the method, when carried out as directed, is specific for inorganic phosphorus. It is based upon the specific reduction by stannous chloride of phosphomolybdic acid in the presence of a definite concentration of the reagents. Heat is not used, for the reaction is completed instantaneously at room temperature. In this method the isolation of phosphorus by precipitation, as performed by Leiboff (2) and also by Samson (3) in their recent methods, is unnecessary. The method of Elek (4), a modification of the gravimetric determination of phosphorus by Pregl (5), is excellent but requires from 3 to 6 mg. of substance containing from 0.2 to 0.5 mg. of phosphorus.

Kuttner and Cohen found the intensity of the blue colored mixture (colloidal reduced oxides of molybdenum) to be proportional to the quantity of phosphorus present. This we are able to corroborate. With 0.005 to 0.015 mg. of phosphate phosphorus in a final mixture of 5 cc., the color is sufficiently intense for the

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plunger type colorimeter. With the comparator type, amounts between 0.0005 and 0.005 mg. can be estimated, the degree of accuracy corresponding to the type of instrument used. The method is rapid and also suitable for minute amounts of phosphorus too small to be estimated by titration or weighing.

The optimum range in the concentrations of both sulfuric acid and sodium molybdate is indicated in the Kuttner-Cohen method (1). It was pointed out by the authors in studying the molybdic acid-stannous chloride reagent, that an excess of acid has the effect of inhibiting the color, while an excess of sodium molybdate tends to increase its intensity and to bring out a color

TABLE I.

Effect of Varying the Concentration of Stannous Chloride.

Stannous chloride added.	Colorimeter readings.	Deviation from average.
cc.	*mm.*	*per cent*
0.5	155	+2.6
0.6	156	+3.3
0.7	154	+2.0
0.8	149	−1.3
0.9	151	0
1.0	150	−0.6
1.0	151	0
1.1	150	−0.6
1.2	150	−0.6
1.3	148	−2.0
1.4	148	−2.0
1.5	149	−1.3

in the blank. Nevertheless, the concentration of both acid and molybdate employed in the phosphorus determination permits sufficient margin of safety so that chance variations in the precision of measurement of the quantities of reagents will be without significant effect, while gross errors alone (10 per cent or more) will cause the appearance of a blue color in the blank or diminish the intensity of the color due to phosphorus. Larger amounts of sodium molybdate can be used provided sufficient sulfuric acid is added. While the intensity of the color is thereby somewhat increased, it is less practical to use the greater concentration because the non-changing zone for color production is then smaller.

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In the use of this method Roe and Kahn (6) have had erroneous results. Concerning the stannous chloride reagent they state in their last paper that, "In using this reagent, which lacks specificity for phosphomolybdic acid, accurate results cannot be obtained unless the amount of the reagent introduced into the standard and unknown solutions is very accurately measured. . . . variations of 0.1 cc. in the addition of this reagent gave errors in excess of 10 per cent." On the other hand Raymond and Levene ((7) p. 623, (8)) have found the method "to be specific for inorganic phosphate." They use a stannous chloride reagent increased in concentration by 50 per cent and obtain results that check between 1 and 2 per cent ((7) p. 627). Levene and Raymond's results we were able to confirm. With stannous chloride stock solution, not aged, variations in amounts up to 50 per cent in excess or less than that originally specified have no demonstrable influence on color production. This is shown in Table I, which gives the colorimeter readings of a series of tubes each containing 0.02 mg. of phosphorus in a total volume of 10 cc., but containing varying amounts of stannous chloride. This experiment was repeated several times with similar results. It is seen that the average deviation from the mean is ± 1.3 per cent. Variations are greater only with stannous chloride stock solution aged several months.

The statement of 0.02 to 0.022 per cent of stannous chloride reagent being the optimal zone for color production was too conservative and can be extended to 0.01–0.03 per cent.

The effect of lowering the temperature on color production was noted. Our attention was directed to this effect by the application of the method by Raymond and Levene (7, 8) to phosphorus estimation in the cold in yeast fermentation mixtures. We found that low temperatures retard development of maximal color production. Below room temperature, the length of time for the blue color to appear and for the maximal coloration to develop is increased progressively as the temperature is lowered. Thus with a temperature of 7° or less the color does not begin to develop for 60 to 90 minutes. Immediate coloration is produced when the tubes attain room temperature upon removal from the cold. Even in the cold, when a reagent is used containing an increase of 5 per cent of sodium molybdate and sulfuric acid the color is developed

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after adding stannous chloride in about 1 to 2 minutes. It is assumed that the unknown and standard are treated exactly alike. Since the reaction takes place without the application of heat and in the cold, as well, the procedure may be extended to the estimation of inorganic phosphorus in cold extracts of muscle, for example.

The description of the method as we have used it follows. The solutions necessary are:

1. Molybdic-Sulfuric Acid Mixture.

(*a*). *10 N H_2SO_4*, or the following which is nearly 10 N and will give satisfactory results. Pour 282 cc. of concentrated H_2SO_4 (95 per cent, sp. gr. 1.84) into about 600 cc. of distilled water, cool, transfer to a liter flask, and make up to volume with distilled water.

(*b*). *7.5 Per Cent Solution of Sodium Molybdate.*—Dissolve 7.5 gm. of Kahlbaum "Zur Analyse" or Eimer and Amend C.P. sodium molybdate in water in a 100 cc. graduated flask and make up to volume with distilled water. The products mentioned have been found reliable. Sodium molybdate obtained from other sources when used to prepare the reagent frequently gives a blue color to the blank test. This is due to soluble tungsten salts or to a substance, containing iron, tungsten, and silica, insoluble in water but soluble in sulfuric acid. If only the latter impurity is present, it will separate out and can be removed by decantation or centrifugation.

These solutions will keep indefinitely in glass-stoppered bottles. The molybdic-sulfuric acid mixture as described by Kuttner and Cohen will keep for several months. We prefer, however, to keep the sulfuric acid, 10 N, and sodium molybdate, 7.5 per cent, in separate containers as stock solutions. If only an occasional determination is made, they are added separately to the phosphate in a manner to be described below. When many determinations are necessary, the solutions may be combined for convenience as follows:

1 volume of 10 N sulfuric acid is diluted with 2 volumes of distilled water. Allow to cool, if necessary, and pour into 1 volume of 7.5 per cent sodium molybdate. Although the mixture thus prepared will keep several months, we prefer to use it freshly made.

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The alternative method of preparing the mixed reagent, as proposed in the original paper, by dissolving the sodium molybdate salt in 2.5 N H_2SO_4 has been rejected.

2. Stannous Chloride Stock Solution.

This is 40 per cent stannous chloride in concentrated hydrochloric acid. Dissolve 10 gm. of stannous chloride in 25 cc. of HCl. Smaller quantities may be prepared if desired. Store in brown glass-stoppered bottle and keep away from heat. We prefer to prepare the stock solution every 4 to 6 weeks although it may keep longer. For immediate use only, dilute 1 part of stock solution in 200 parts of distilled water. Any unused portions should be discarded.

3. Standard Phosphate Stock Solution.

Dissolve 0.4394 gm. of dried monopotassium phosphate in 1 liter of distilled water, and add a few drops of chloroform to prevent mold formation. 1 cc. = 0.1 mg. of phosphorus. Make two standard phosphate solutions by diluting 3 cc. and 6 cc. in 100 cc. graduated flasks and fill to the mark with water. The solutions contain 0.003 and 0.006 mg. of phosphorus per cc.

The solution to be tested should contain between 0.25 and 1.0 mg. of phosphorus per 100 cc. Proceed in the following manner. (1) Transfer 5 cc. of the sample to be examined to a test-tube graduated at 5 and 10 cc. The sample may contain between 0.01 and 0.05 mg. of phosphorus. (2) Place 5 cc. of each standard phosphate solution in two other similarly graduated test-tubes so that one tube contains 0.015 mg. and the other 0.03 mg. of phosphorus. (3) Add to each tube 4 cc. of the mixed molybdic-sulfuric acid reagent and mix. As an alternative procedure add to each tube 1 cc. of 10 N H_2SO_4, 2 cc. of distilled water (mix contents), and 1 cc. of 7.5 per cent sodium molybdate solution. Close with a rubber stopper and invert. (4) Then add 1 cc. of the diluted stannous chloride reagent to each tube. Immediately invert once or twice. The colors can be compared at once or within the next 2 hours in the usual manner according to the type of instrument used.

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COMPOSITION OF BONE.

IX. EQUILIBRATION OF SERUM WITH DICALCIUM PHOSPHATE.*

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The mechanism of calcification is still obscure. One theory (2) postulates the formation of intermediate calcium-protein and calcium-protein-phosphate compounds, produced by the interaction between the cartilage protein and the calcium and phosphorus of the body fluids; the calcium-protein-phosphate compound is supposed to decompose with the formation of calcium phosphate and the release of the protein. The other leading theory ascribes calcification to the precipitation of $Ca_3(PO_4)_2$ and $CaCO_3$.

A number of lines of evidence point to precipitation of calcium phosphate as a likely mechanism. Thus the correlation between normal calcification, healing rickets, and active rickets on the one hand, and the empirical Ca × P product of Howland and Kramer (3) on the other, is suggestive of the operation of a solubility product. Furthermore, experiments on calcification *in vitro* (4, 5) show that calcification may be produced or prevented at will merely by changing the concentrations of calcium and phosphorus in the inorganic serum solution in which the calcifying tissue is immersed. Here, too, the occurrence of calcification and the Ca × P product are related in such a way as to suggest the precipitation of calcium phosphate. The inhibitory effect of magnesium on *in vitro* calcification (6–8) is overcome by increased concentration of phosphate; increase of the ionic strength of the solutions also inhibits calcification *in vitro* (7, 9). These factors

* A preliminary report (1) was read before the Society for Experimental Biology and Medicine, New York, October 16, 1929.

affect calcification in a manner analogous to their effect on the precipitation of calcium phosphate.

However, physicochemical studies (10–26) have not borne out the view that calcification is due to the precipitation of $Ca_3(PO_4)_2$. Thus, Holt, La Mer, and Chown (10) and Holt (11) were led to the conclusion that even ricketic serum is supersaturated with respect to $Ca_3(PO_4)_2$. Sendroy and Hastings (12) concluded that serum does not appear to be supersaturated with respect to $Ca_3(PO_4)_2$ "in the usual sense of the term" and that supersaturation cannot be the sole explanation for the apparently abnormal amounts of calcium in serum. Klinke's (13) experiments constitute additional evidence against the view that serum is supersaturated with respect to $Ca_3(PO_4)_2$.

It might therefore be concluded that the precipitation theory of calcification is untenable. But before abandoning this theory, another alternative remains to be investigated; namely, the possible bearing of $CaHPO_4$ on this problem.

We realize, of course, that the presence of $CaHPO_4$ in bones has never been demonstrated (14, 15). Nevertheless, it is of interest to obtain data on the solubility relations of $CaHPO_4$ to see what light they can throw on the mechanism of calcification.

Because the proteins present in serum give rise to complications, we studied the solubility equilibria of $CaHPO_4$ in protein-free solutions first. It was found (15) that solutions with the inorganic composition of ricketic blood serum are markedly undersaturated with respect to $CaHPO_4$ and that it is only solutions which have Ca × P products greater than about 50 which are supersaturated with respect to this compound.

It had been pointed out previously (14, 15) that the ion product $[Ca^{++}] \times [HPO_4^{=}]$ in a serum with a given calcium and phosphorus content cannot be greater than the ion product in an inorganic serum solution with the same calcium and phosphorus content. If the value of this ion product is different it must be less in serum, since part of the serum calcium appears to be bound to protein, thus making $[Ca^{++}]$ in serum less than [Ca]. On the basis of these considerations it appeared that blood sera with Ca × P products of 50 or less are undersaturated with respect to $CaHPO_4$.

The present paper is a report of experiments in which blood sera were shaken with crystalline $CaHPO_4$.

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Methods.

Calcium and phosphorus[1] were determined in duplicate as described previously (15). The $CaHPO_4$ used was the same analyzed crystalline[2] preparation used in the previous experiments (15). The new Hastings hydrogen ion colorimeter (Bausch and Lomb) was employed for the determination of pH. The equilibration was carried out at 38° ± 2°. Total nitrogen and non-protein nitrogen were determined according to the method of Van Slyke (27).

Calculations.—The calculation of $[HPO_4^{=}]$ is the same[3] as that employed for protein-free solutions (14, 15). [Ca] is the total calcium as given by analysis; the symbol $[Ca^{++}]$ represents the concentration of calcium ions.

Calf Serum.

Serum from the blood of a 6 months old calf was analyzed in duplicate for calcium, phosphorus, and pH. Two 20 cc. aliquots of the serum were shaken with $CaHPO_4$ for 1 hour as previously described; two other aliquots were shaken for 2 hours. The results are given in Table I.

Our previous experiments, in which protein-free serum solutions were shaken with $CaHPO_4$, showed that equilibrium was attained after shaking for only 1 hour; the equilibrium constant obtained did not change during the subsequent 3 hours. Serum similarly comes rapidly into equilibrium with $CaHPO_4$.

[1] In this paper "phosphorus" means the inorganic phosphorus of the serum.

[2] These crystals of $CaHPO_4$ had been dried at 110° for 7 hours. The analytical data showed that the specimen so prepared contained 1.87 molecules of water of crystallization for every molecule of $CaHPO_4$.

[3] This depends on the assumption that the total inorganic phosphorus as obtained by analysis is present as phosphate ions. This appears to be a valid assumption since numerous investigators have found that the inorganic phosphorus in serum is 100 per cent dialyzable. Grollman (28) found that addition of large amounts of calcium to serum reduced the relative amount of filtrable phosphorus. However, for mammalian serum he found that the phosphorus begins to be held back only when the empirical Ca × P product exceeds 95. In all of the sera studied here the Ca × P products were less than 90.

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TABLE I.

Sera Equilibrated with $CaHPO_4$ at 38°.

Specimen.*	[Ca]		[P]		Final pH.	$[HPO_4^=]$		$[Ca] \times [HPO_4^=]$	
	Initial.	Final.	Initial.	Final.		Initial.	Final.	Initial.	Final.
Calf serum.									
	mM	*mM*	*mM*	*mM*		*mM*	*mM*	$\times 10^6$	$\times 10^6$
1 hr. (a)	2.64	2.52	2.42	2.87					
	2.59	2.52	2.36	2.68					
	2.62	2.52	2.39	2.78	7.50	1.98	2.41	5.2	6.1
1 " (b)	Same.	2.42	Same.	2.80					
		2.44		2.84					
		2.43		2.82	7.53	1.98	2.50	5.2	6.1
2 hrs. (a)	Same.	2.34	Same.	2.93					
		2.37		2.84					
		2.36		2.89	7.48	1.98	2.51	5.2	5.9
2 " (b)	Same.	2.39	Same.	2.87					
		2.42		2.80					
		2.41		2.84	7.49	1.98	2.47	5.2	6.0
Lamb sera equilibrated for 1 hr.†									
Serum 1.	2.62	2.65	2.29	2.45	7.58	1.90	2.18	5.0	5.8
" 2.	2.34	2.32	2.90	3.00	7.55	2.41	2.67	5.6	6.2
" 3.	2.47	2.44	2.35	2.58	7.57	1.95	2.29	4.8	5.6
" 4.	2.52	2.59	2.35	2.55	7.49	1.95	2.21	4.9	5.7
Human sera equilibrated for 1 hr.†									
Cord 1.	2.77	2.87	1.77	2.06	8.4	1.47	2.02	4.1	5.8
" 2.	2.87	2.87	1.58	1.84	7.85	1.31	1.73	3.8	5.0
" 3. (a)	2.92	2.94	1.94	2.10	7.58	1.61	1.87	4.7	5.5
" 3. (b)	2.92	2.87	1.94	2.06	7.63	1.61	1.85	4.7	5.3
Adult cardiac. (a)	2.42	2.69	1.74	2.45	8.03	1.44	2.35	3.5	6.3
" " (b)	2.42	2.69	1.74	2.32	8.06	1.44	2.33	3.5	6.0
Normal adult. (a)	2.50	3.19	1.13	2.16	7.62	0.94	1.92	2.4	6.1
" " (b)	2.50	3.22	1.13	2.19	7.53	0.94	1.93	2.4	6.2

* (a) and (b) indicate duplicate experiments.

† The values given for calcium and phosphorus are the averages of two determinations (*cf.* calf serum).

There is excellent agreement between the duplicate experiments at 1 hour of equilibration; the same holds true for the duplicate experiments at 2 hours. Such equilibration experiments with serum are therefore accurately reproducible. There is no appreciable change in the [Ca] $\times$ [$HPO_4^=$] products after the 1st hour. In all the subsequent experiments, therefore, the sera were shaken for only 1 hour.

After the serum was shaken for 1 hour the calcium decreased about 5 per cent; the phosphorus, however, increased about 16 per cent. In the duplicate experiment the calcium decreased about 7 per cent while the phosphorus increased about 19 per cent. After the serum was shaken for 2 hours, the calcium decreased 9.5 per cent; the phosphorus, however, increased about 22 per cent. The duplicate 2 hour experiment gave results for calcium and phosphorus that were almost identical with those of the duplicate 1 hour experiment. Equilibration had resulted in a definite increase in the product [Ca] $\times$ [$HPO_4^=$] from 5.2×10^{-6} to $(6.0 \pm 0.1) \times 10^{-6}$.

Lamb Serum.

In this set of experiments the blood sera of four lambs, all of them 6 months old, were employed. The results are given in Table I.

For the first three sera, the calcium values after equilibration were the same, within the analytical error, as the initial values; in Lamb Serum 4 there was a small but definite increase in calcium. The phosphorus value in each case increased slightly but definitely as a result of equilibration.

The [Ca] $\times$ [$HPO_4^=$] products in the sera as drawn were unusually high; these sera were obtained from healthy young animals at an age when calcification is occurring vigorously. In spite of the high concentrations of calcium and phosphorus present initially, these sera were undersaturated with respect to $CaHPO_4$. This is shown by the increase in the [Ca] $\times$ [$HPO_4^=$] products following equilibration.

Human Serum.

The first three specimens were taken from the cord at delivery. They show little change in calcium and a definite increase in

phosphorus as a result of shaking with $CaHPO_4$. This increase is also shown by the increase in the $[Ca] \times [HPO_4^=]$ products. (See Table I.)

In three cases the equilibration was performed in duplicate; these are indicated by the letters (a) and (b) in Table I. The sera were analyzed for calcium and phosphorus in duplicate, but only the mean values are given in Table I; the agreement between duplicate analyses of the same serum was the same as that shown in the experiments on calf serum.

Equilibration produced an 11 per cent increase in calcium and a 41 per cent increase in phosphorus in the serum of an adult cardiac. The product $[Ca] \times [HPO_4^=]$ increased markedly from 3.5×10^{-6} to 6.3×10^{-6}. A duplicate experiment gave similar results.

The most striking change was obtained with the serum from a normal adult. The final calcium was 28 per cent greater than the initial value, and the final phosphorus was 91 per cent greater than in the serum as drawn. The $[Ca] \times [HPO_4^=]$ product showed a striking increase from 2.4×10^{-6} to 6.1×10^{-6}. A duplicate experiment gave similar results.

Effect of Shaking on Inorganic Phosphorus.

The question arose as to whether mere shaking for 1 hour at 38°, in the absence of the solid phase, might not produce an increase in the inorganic phosphorus concentration of serum. This appeared improbable for many reasons; but even if it did occur, it would not affect the conclusions drawn. No matter what the origin of the additional inorganic phosphorus, the fact that the sera were capable of retaining this increased concentration of phosphorus with an accompanying increase in the value of the $[Ca] \times [HPO_4^=]$ product in the presence of the solid phase, showed that the sera were initially undersaturated with respect to $CaHPO_4$. Certainly the calcium concentrations could not increase, as they did in the adult human sera, in the face of such marked increases in phosphorus concentration unless the sera were definitely undersaturated with respect to $CaHPO_4$.

Nevertheless, to rule out whatever doubt remained on this point the following experiment was performed. A specimen of adult human serum was divided into four aliquots. One aliquot was analyzed for inorganic phosphorus. The other three aliquots

were then put into the thermostat. One aliquot was shaken with $CaHPO_4$ for 1 hour at 38°; another aliquot was shaken for 1 hour at 38° without the addition of the solid phase. A fourth aliquot was left undisturbed in the thermostat for 1 hour at 38° without the addition of any solid phase and without shaking. They were then all analyzed at the same time for calcium and inorganic phosphorus. The results are given in Table II.

TABLE II.

Effect of Shaking on Inorganic Phosphorus.

Adult human Serum 3.

Aliquot.	Treatment.	Ca	P
		mg. per cent	*mg. per cent*
a	None.		3.4
			3.2
			3.3
b	1 hr. at 38°. No $CaHPO_4$. No shaking.	9.8	3.3
		9.9	3.4
		9.9	3.4
c	1 " " 38°. " " Shaking.	9.8	3.3
		9.8	3.4
		9.8	3.4
d	1 " " 38°. Shaking with $CaHPO_4$.	11.4	5.2
		11.3	5.0
		11.4	5.1

It is seen that standing for 1 hour at 38° does not result in an increase in the inorganic phosphorus. Neither does shaking without the solid phase for 1 hour result in any rise in the inorganic phosphorus concentration. Shaking with $CaHPO_4$, however, produces a striking increase in the phosphorus concentration from 3.4 mg. per cent to 5.1 mg. per cent. At the same time there is a definite increase in the calcium content from 9.9 mg. per cent to 11.4 mg. per cent.

This shows unmistakably that the increase in the phosphorus concentration, as well as the increase in the calcium concentration, is due to the presence of $CaHPO_4$.

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pH Change Produced by Equilibration.

The specimens of blood were obtained under conditions which rendered it impossible to avoid the loss of some CO_2. The initial pH was therefore not determined; it was assumed to be 7.35 in all the sera (29).

In a number of instances one aliquot of the serum was stoppered and set aside without the addition of $CaHPO_4$. After equilibration of the other aliquot, the pH of the equilibrated and non-equilibrated aliquots were determined at the same time. Table III shows that the sera not equilibrated were all somewhat more alkaline than pH 7.35, due in all probability to loss of some CO_2.

TABLE III.

pH Change in Serum Produced by Equilibration with $CaHPO_4$ for 1 Hour.

Serum.	Not equilibrated.	Equilibrated.*
Calf.	7.79	(a) 7.50
"	7.79	(b) 7.53
Cord 1.	8.08	(a) 8.03
" 1.	8.08	(b) 8.06
Adult cardiac.	7.66	(a) 7.58
" "	7.66	(b) 7.63
Normal adult.	7.68	(a) 7.62
" "	7.68	(b) 7.53

* (a) and (b) indicate duplicate experiments.

Equilibration with $CaHPO_4$ produced very little change in pH as is seen from Table III; there was in each case a slight change towards the acid side. However, in no case was the change sufficient to render the serum more acid than pH 7.50.

The pH values given in Table I are those obtained for the various sera after equilibration. It will be noted that the pH in no case is lower than 7.48. Thus after equilibration in no instance is the pH value more acid than normal serum (29). The increased concentrations of calcium and phosphorus therefore cannot be due to an increase in the solvent power of the sera resulting from a pH more acid than normal.

DISCUSSION.

These experiments were performed to determine whether shaking serum with $CaHPO_4$ for brief periods would result in an increase or a decrease in the concentrations of calcium and phosphorus. Since there resulted in each case an increase in the phosphorus concentration and in some cases an increase in the calcium concentration as well, such that in all sera the $[Ca] \times [HPO_4^=]$ products increased as a result of equilibration, it appears evident that serum is undersaturated with respect to $CaHPO_4$. Furthermore, the lower the initial products, the greater is the degree of undersaturation.

Mond and Netter (23) shook bovine serum, containing 9.4 mg. per cent calcium, with $CaHPO_4$. At the end of 1 hour the serum calcium was unchanged. After the serum was shaken for 3 hours the calcium fell to 8.6 mg. per cent. At the end of $6\frac{1}{2}$ hours it had fallen to 8.1 mg. per cent. These authors concluded that serum was supersaturated with respect to $CaHPO_4$ and that when serum is shaken with solid $CaHPO_4$, a small quantity of $CaHPO_4$ gradually precipitates out.

It may be pointed out that shaking with $CaHPO_4$ can produce a decrease in the calcium concentration without a simultaneous decrease in phosphorus. In fact the phosphorus may increase so that the $[Ca] \times [HPO_4^=]$ product is greater after equilibration than in the serum initially. This is shown clearly by the data on calf serum in Table I.

Calcium values alone, therefore, do not provide sufficient information on which to base conclusions regarding the occurrence of precipitation, or the existence of supersaturation. Since phosphorus values and pH values are both lacking in the paper of Mond and Netter, $[Ca] \times [HPO_4^=]$ cannot be calculated from their data.

Kleinman (25) also equilibrated serum with $CaHPO_4$. He concluded "Tabelle xx zeigt, dass sekundäres Calciumphosphat als Bodenkörper wirkungslos ist." However an inspection of his Table xx shows that although the calcium had not changed, the phosphorus had increased slightly but definitely. Kleinman had done his four experiments with one serum specimen; apparently that particular serum was only very slightly undersaturated with respect to $CaHPO_4$. In the light of our results with Lamb Serum

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2 (Table I), it is readily understood how, if our equilibration experiments had all been performed with that serum alone, we also might have concluded that $CaHPO_4$ is without effect.

Nitschke and Freyschmidt (21) considered serum to be saturated with respect to $CaHPO_4$. In a later paper, Nitschke (22) concluded from dialysis experiments that, in serum which contains 10 mg. per cent calcium and 3.7 mg. per cent phosphorus, about 2 mg. per cent calcium is bound to protein, about 5.6 mg. per cent is in the form of calcium ions, and that about 2.4 mg. per cent calcium exists as "molecular" $CaHPO_4$. He also concluded "Die auf dieselbe Weise bestimmte Gleichgewichtskonstante für übersättigte Calciumphosphatlösungen beträgt für die Gleichung"

$$[Ca^{++}] \times [HPO_4^{=}] = K = 67 \times 10^{-7}$$

Nitschke did not perform any equilibration experiments with solid $CaHPO_4$. Inspection of his data shows that his solutions had a composition analogous to those of our solutions which were undersaturated as evidenced by equilibration with solid $CaHPO_4$ (15).

Peters and Eiserson (30) studied the relative effects of phosphorus and protein on serum calcium. Following a suggestion of Professor A. B. Hastings, these authors attempted to correlate their data with the solubility of $CaHPO_4$ as follows: "If the solubility product of $CaHPO_4$ were the factor responsible for the effect of P on Ca, then the curve of ionized Ca and HPO_4 should be a right angle hyperbola and the curve relating their logarithms should be a 45° straight line."

It seems to us that this should hold true only if sera were just saturated with respect to $CaHPO_4$. If sera were exactly saturated with respect to $CaHPO_4$ then

$$[Ca^{++}] \times [HPO_4^{=}] = K'_{s.p.\ CaHPO_4}$$

Graphically this is given by a rectangular hyperbola since it is of the form xy = constant. However, our experiments show that serum is *undersaturated* with respect to $CaHPO_4$ and that the degree of undersaturation varies from serum to serum. The ion product $[Ca^{++}] \times [HPO_4^{=}]$ in serum therefore cannot be a constant.

Peters and Eiserson applied this criterion and concluded

"The results were not satisfactory. . . . This does not prove that the solubility product of $CaHPO_4$ is not the factor responsible for the effect of P on serum Ca. It may only mean that the calculations involved in the indirect estimation of HPO_4 were not sufficiently accurate."

We wish to point out that even if $[HPO_4^{=}]$ were obtained accurately from direct determinations of pH, and even if a method were available for obtaining $[Ca^{++}]$ accurately, still the curve relating their logarithms should not be a 45° straight line for serum as drawn. This relationship should be expected to hold only in sera exactly saturated with $CaHPO_4$.

Calcium Ion Concentration in Serum.

Calcium is essential for calcification. However, the form in which calcium is present is as important as the total amount of calcium given by analysis. Thus Shipley, Kramer, and Howland (5), in their experiments on calcification *in vitro*, found that when the calcium was present as chloride, acetate, or lactate, calcification was prompt and thorough; when the calcium was present as citrate no calcification was obtained.

Recent conductivity studies (31, 32) showed that the citrate ions combine with the calcium ions to form an unionized complex; the presence of citrate causes a marked diminution in the calcium ion concentration. It is thus seen that citrate inhibits calcification because it decreases the calcium ion concentration; for calcification to occur, the calcium must be present as calcium ion.

There are no methods in the literature for the accurate determination or calculation of $[Ca^{++}]$ in individual specimens of serum. Until an accurate direct method is devised, the concentration of ionized calcium in serum can be obtained only approximately from indirect estimations. It is of interest in this connection to note that the data obtained in the equilibration experiments with $CaHPO_4$ may be utilized for calculating the approximate value of the ionized and bound calcium in serum. At present we do not know how valid are the assumptions involved in such a calculation; the values of the calcium ion concentration so obtained are therefore of undeterminable accuracy. However, the results obtained with this new method are in agreement with the values for calcium ion concentration obtained by other investigators who used totally different methods.

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After inorganic serum solutions were shaken with $CaHPO_4$ for 1 hour at 38°, the product $[Ca^{++}] \times [HPO_4^{=}]$ was a constant $(3.4 \pm 0.1) \times 10^{-6}$. In serum, similarly shaken with $CaHPO_4$, the product $[Ca] \times [HPO_4^{=}]$ was not a constant. Although duplicate experiments gave results which were in good agreement, the product varied from serum to serum; the values ranged from 5.0×10^{-6} to 6.3×10^{-6}. All the products were greater than 3.4×10^{-6}, the value for inorganic serum solutions.

Since equilibrium had apparently been established between the solid $CaHPO_4$ and the sera, the ion product $[Ca^{++}] \times [HPO_4^{=}]$ in the sera should be the same as in inorganic serum solutions in equilibrium with $CaHPO_4$; *i.e.*, 3.4×10^{-6}. The products $[Ca] \times [HPO_4^{=}]$ were greater than $K'_{s.p.}CaHPO_4$. This indicates that part of the total calcium was "bound" in a form which rendered it unavailable for this ionic equilibrium.

In the equilibrated serum, the calcium ion concentration should be given by

$$[Ca^{++}]_{equil.} = \frac{K'_{s.p.}\ CaHPO_4}{[HPO_4^{=}]_{equil.}} \tag{1}$$

and the bound calcium, [CaX], should be given by

$$[CaX]_{equil.} = [Ca]_{equil.} - [Ca^{++}]_{equil.} \tag{2}$$

where [CaX] is the concentration of bound calcium and [Ca] is the concentration of total calcium after equilibration.

The assumption is next made that the concentration of bound calcium is the same before and after equilibration. This would appear to be a valid assumption in those cases in which equilibration produces little or no change in the total calcium and in pH.[4]

The concentration of ionized calcium in the serum as drawn can then be obtained from the expression

$$[Ca^{++}]_{serum} = [Ca]_{serum} - [CaX]_{serum} \tag{3}$$

where

$[Ca^{++}]$ = concentration of ionized Ca in serum as drawn.
$[Ca]$ = " " total " " " " " "
$[CaX]$ = " " bound " " " " " "

[4] In those cases where there has been a change in pH, the amount of calcium bound at pH 7.4 may be calculated from the amount bound at the pH of equilibrated serum by means of an expression derived from Equation 72 of Hastings, Murray, and Sendroy (24).

TABLE IV.

Concentration of Ionized Calcium in Serum.

Specimen. (1)	Total N. (2)	Non-protein N. (3)	Protein. (4)	Total initial Ca. (5)	Bound Ca.* At equilibrium. (6)	Bound Ca.* At pH 7.4. (7)	Initial Ca^{++}. (8)	$\frac{Ca^{++}}{Total\ Ca} \times 100$. (9)	$\frac{Bound\ Ca}{Protein}$. (10)
	gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	*mg. per gm.*
Calf.	1.05	0.033							
	1.04	0.031	6.4	10.5	(a) 4.5	4.2	6.3	60	0.66
	1.05	0.032			(b) 4.3	3.9	6.6	63	0.61
Lamb 1.	1.11	0.046							
	1.09	0.041	6.6	10.5	4.4	3.9	6.6	63	0.59
	1.10	0.044							
" 2.	1.21	0.029							
	1.18	0.041	7.3	9.4	4.2	3.8	5.6	60	0.52
	1.20	0.035							
" 3.	1.27	0.028							
	1.37	0.027	8.1	9.9	3.9	3.5	6.4	65	0.43
	1.32	0.028							
" 4.	1.39	0.037							
	1.31	0.025	8.3	10.1	4.2	4.0	6.1	60	0.48
	1.35	0.031							
Human cord 1.	1.16	0.043							
	1.16	0.043	7.0	11.1	4.8	2.8	8.3	75	0.40
	1.16	0.043							
" " 2.	1.02								
	1.09	0.050	6.3	11.5	3.7	2.8	8.7	75	0.44
	1.06								
" " 3.	1.11								
	1.13	0.063	6.6	11.7	(a) 4.5	4.0	7.7	66	0.61
	1.12				(b) 4.1	3.5	8.2	70	0.53
Adult cardiac.	1.47								
	1.45	0.248	7.6	9.7	(a) 5.0	3.5	6.2	64	0.46
	1.46				(b) 4.7	3.2	6.5	67	0.42
Normal adult.	1.38								
	1.42	0.053	8.4	10.0	(a) 5.7	4.9	5.1	51	0.58
	1.40				(b) 5.9	5.4	4.6	46	0.64
Average.....							6.6	63	0.53

* (a) and (b) indicate duplicate experiments.

The results obtained by the aid of such calculations are given in Table IV. In order to see whether there is any regular relationship between the concentration of CaX when calculated in this way and the serum protein concentration, the sera were analyzed for total nitrogen and non-protein nitrogen. Column 4 gives the protein content in gm. per 100 cc. The total calcium in the original sera is given in Column 5. The bound calcium CaX, in the serum at equilibrium is given in Column 6; the calcium bound at pH 7.4 was calculated from the values in Column 6 by means of the expression

$$[CaX]_{serum} = [CaX]_{equil.} \times \frac{7.4 - 6.0}{pH_{equil.} - 6.0} \quad (4)$$

which was derived from Equation 72 of Hastings, Murray, and Sendroy (24). These values are given in Column 7. The calcium ion concentrations in the original serum calculated with the aid of these four equations are given in Column 8. Column 9 gives the per cent of total calcium which is present in the original sera in ionized form. All of the concentrations in Table IV are expressed in terms of mg. and gm. per 100 cc.

In order to see what relation exists between the protein concentration and the concentration of bound calcium, the data in Column 10 were calculated. This column gives the ratio of mg. of bound Ca per gm. of serum protein.

It will be seen from Table IV that the values for ionized calcium give an average of 6.6 mg. per 100 cc., and that the average ratio of bound Ca: protein is 0.53 mg. per gm. These results may be formulated as follows:

$$Ca = 0.53 \times \text{protein} + 6.6 \quad (5)$$

This is quite similar to the results obtained by other investigators. Hastings, Murray, and Sendroy plotted total calcium against protein and obtained an expression which may be written[5]

$$Ca = 0.56 \times \text{protein} + 5.6 \quad (6)$$

Peters and Eiserson, using the same statistical method, obtained

$$Ca = 0.56 \times \text{protein} + 6.0 \quad (7)$$

[5] See reference (30), footnote on page 158.

Marrack and Thacker (19) applied a similar method to data on various body fluids and obtained three straight lines whose equations may be written

$$Ca = 0.66 \times \text{protein} + 6.0 \quad (8)$$
$$Ca = 0.61 \times \text{protein} + 5.5 \quad (9)$$
$$Ca = 0.55 \times \text{protein} + 5.0 \quad (10)$$

It is interesting to note that although the calculation of $[Ca^{++}]$ in serum from $[Ca] \times [HPO_4^{=}]$ involves a number of assumptions, the results obtained are in agreement with those obtained by other indirect methods. According to Column 9 of Table IV about 60 per cent of the calcium in serum is present as calcium ion; the values range from 46 per cent to 75 per cent. These values are similar to those obtained by numerous investigators who employed dialysis and ultrafiltration methods for estimating the concentration of diffusible calcium in serum.[6]

CONCLUSION.

These equilibration experiments show that serum is undersaturated with respect to $CaHPO_4$. In normal young animals, in whom calcification is proceeding vigorously, the calcium and inorganic phosphorus concentrations in the serum may reach high levels; such sera are only slightly undersaturated with respect to $CaHPO_4$. Sera which contain concentrations of Ca and P such as occur in normal adult animals or in ricketic young animals are markedly undersaturated with respect to $CaHPO_4$.

It may be remarked here that the concentrations of calcium and of phosphorus in serum are not the only factors which are involved in calcification. The conditions existing locally in the tissues must be understood before we can have a complete description of the mechanism of calcification.

[6] These results should not be construed as demonstrating that all of the bound calcium is tied to protein. At present it is impossible to determine what percentage of serum calcium is bound to protein, and how much is bound to other unknown factors. If the values for $[Ca^{++}]$ obtained by the use of Equations 1 to 4 are correct, then the difference between the total and ionized calcium gives the concentration of bound calcium. It does not, however, give any information as to the form in which the bound calcium exists. The ratio bound Ca: protein was calculated merely for the sake of comparison with the results obtained by other investigators who employed entirely different methods in calculating this ratio.

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With a given state of affairs in the provisional zone of calcification, the occurrence of calcification will depend on the chemistry of the serum. On the other hand, with a given state of affairs in the chemistry of the serum, the occurrence of calcification will depend on the local factors.[7] When rickets heals, calcification does not occur simultaneously in all regions of the affected areas; this indicates that there may be marked differences in the local factors in different parts of the same bone. Thus Hess, Weinstock, Rivkin, and Gross (35) report the experimental production of a type of rickets which persists in the face of high concentrations of serum calcium and phosphorus. They attribute this to highly adverse local conditions.

The composition of bone is not known with certainty. On the one hand, the main constituent is generally stated to be $Ca_3(PO_4)_2$ or a compound with the formula $3Ca_3(PO_4)_2 \cdot CaCO_3$, analogous to minerals of the apatite group. However, as pointed out previously (14, 15), neither of these compounds has as yet been demonstrated to be present in bone. Furthermore, solubility studies have as yet not succeeded in showing that such compounds may be obtained by precipitation from body fluids, or that they may exist in equilibrium with body fluids.

On the other hand, neither has the presence of $CaHPO_4$ in bones been demonstrated. Furthermore, it is generally stated that in neutral or alkaline solutions $Ca_3(PO_4)_2$, or a still more basic phosphate, is precipitated and that $CaHPO_4$ is obtainable only from definitely acid solutions. Finally bone analyses give values which more nearly fit such formulas as $Ca_3(PO_4)_2$ or $3Ca_3(PO_4)_2 \cdot CaCO_3$ than $CaHPO_4$.

Yet, $CaHPO_4$ comes rapidly into equilibrium with inorganic serum solutions and with serum. This curious finding raises the question as to whether $K'_{s.p.}CaHPO_4$ is of biological significance.

Miss Dora Luntz and Mr. Jac Siegel gave technical assistance in these experiments.

SUMMARY.

1. Blood sera were shaken with crystalline $CaHPO_4$ for 1 hour and then analyzed for calcium, phosphorus, and pH.

[7] *Cf.* discussion of "Local Factors" by Shear and Kramer (14) pages 139–140. See also Jones (33) and Hess (34) page 138.

2. The product [Ca] × [$HPO_4^=$] increased in each case as a result of equilibration.

3. The lower the initial value of [Ca] × [$HPO_4^=$] in the serum as drawn, the greater was the increase produced by equilibration.

4. These experiments show that serum is undersaturated with respect to $CaHPO_4$.

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THE FORMYLATION OF AMINO ACIDS.

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Resolution of amino acids into their optically active forms by means of alkaloids is in most instances best effected through their formyl derivatives. These important intermediates are prepared from amino acids by the action of formic acid. Water is formed in the reaction.

$$R \cdot \underset{\displaystyle NH_2}{\overset{\displaystyle |}{CH}}—COOH + HCOOH = R \cdot \underset{\displaystyle NH—CHO}{\overset{\displaystyle |}{CH}}—COOH + H_2O$$

The procedure actually in use has been described by Fischer and Warburg.[1] The amino acid is heated for 3 hours on a steam bath with 1.5 parts of anhydrous formic acid. The solution is then concentrated to dryness under reduced pressure and the whole operation repeated twice. This procedure is a rather tedious one. Furthermore, even under these conditions, the reaction is not complete; in fact Fischer and Warburg have assumed it to be an equilibrated one.

The present publication deals with the description of a method permitting the gradual and almost complete removal by distillation of the water formed in the reaction mixture. In a rather short time the reaction proceeds practically to completion. The formyl derivatives of *d,l*-valine, *d,l*-leucine, and *d,l*-phenylalanine were thus obtained in almost quantitative yield.

THEORETICAL.

A mixture of pure amino acid and anhydrous formic acid (in the respective mol proportions of 1 to 6) is refluxed in the flask of

[1] Fischer, E., and Warburg, O., *Ber. chem. Ges.*, **38**, 3998 (1905). See also Fischer, E., *Ber. chem. Ges.*, **39**, 2928 (1906).

the distilling device described below. The removal from the reaction mixture of the water, while the reaction is in progress, is accomplished by distillation in the presence of pure toluene continuously reintroduced into the reaction flask. Since at the same time the toluene removes also practically all the excess of formic acid, there remains in the reaction flask a mixture of molten or crystalline formyl derivative and toluene. Other liquids, such as chloroform, carbon tetrachloride, or benzene, which theoretically are also suited as carriers, do not present the same advantages as toluene. The principal factors which determined the choice of toluene were, first, the great temperature difference between its boiling point and the boiling points of the constant boiling mixtures it is known to form with water and formic acid, and second, the favorable composition of these mixtures. The toluene-water mixture, boiling constant at 84.43° under 760 mm. pressure, contains 19.7 per cent by weight of water (55.6 mol per cent).[2] The toluene-formic acid mixture, boiling constant at 85.8° under 760 mm. pressure, contains 50 per cent by weight of each component[3] (66.7 mol per cent of formic acid). Actually, a composite mixture of these two binary azeotropes distils only for a certain period of time. This is due to the fact that the replacement by toluene of the formic acid, which is gradually removed, finally leads to the formation of two layers in the reaction flask. A distillate of somewhat different, but almost constant composition, is obtained from then on.

EXPERIMENTAL.

Fig. 1 is a photograph and Fig. 2 a diagram of the distilling device which is made entirely of Pyrex glass. Its main feature, the column, is a tube filled with 8 to 9 mm. glass beads,[4] insulated up to the spiral condenser with asbestos cord which is maintained in

[2] As derived from data in Young, S., *J. Chem. Soc.*, **83**, 74 (1903). More recently, Barbaudy, J., *J. chim. physique*, **23**, 298 (1926), found 84.34° and 55.73 mol per cent of water. Golodetz, A., *Chem.-Ztg.*; **36**, 298 (1912) gives 84.1° and 19.6 weight per cent of water. See also Naumann, A., *Ber. chem. Ges.*, **10**, 1427, 2100 (1877).

[3] Lecat, M., La tension de vapeur des mélanges de liquides. L'azéotropisme. Part I, Brussels and Ghent, p. 72, No. 121 (1918).

[4] For this purpose the common stringing beads as used by children and sold in department stores have been found very satisfactory.

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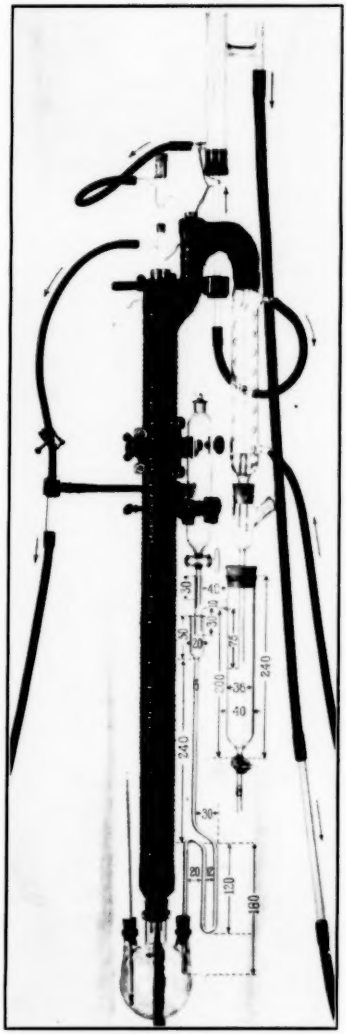

FIG. 1.

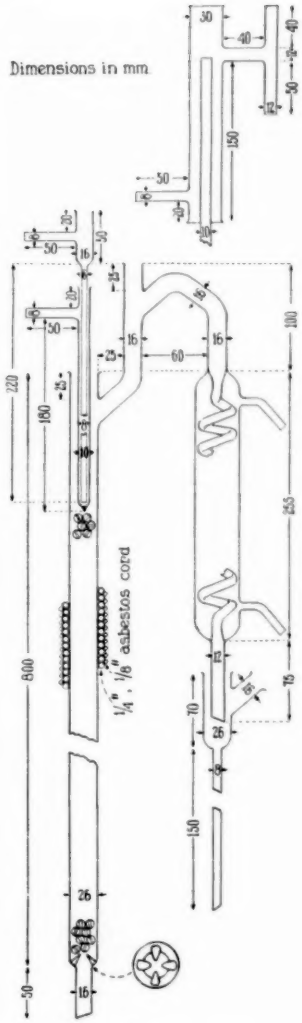

FIG. 2.

FIG. 1. Photograph of the distilling device.
FIG. 2. Diagram of the distilling device illustrated in Fig. 1.

place by means of friction tape. The vapors reaching the upper part of the column are there partly condensed by a small reflux condenser supplied with cooling water from the constant water level reservoir. The rate of flow of the water through the reflux condenser can be regulated by means of a screw clamp and can be observed through the glass tube which encloses the capillary outlet tube connected with the rubber tubing. The condensation temperature of the escaping vapors is read on a short thermometer inserted in the side neck of the column. The vapors are finally condensed in the spiral condenser (a standard Pyrex condenser; jacket length, 10 inches). The distillate drops through a funnel into a separator whose volume up to the level of the outflow is 180 cc. and which has a graduation at 95 cc., slightly below the outlet of the funnel. The side tube of this funnel, through which the filling of the separator is done, constitutes the opening to the air of the otherwise closed system. The bend in the return tube to the reaction flask serves as a trap to prevent the vapors from entering that tube. The stoppers in contact with vapors were cork, well coated outside with cellulose acetate collodion, for they are attacked and shrink; elsewhere rubber stoppers were used. The cork stoppers have to be renewed after each operation. Cleaning of the column and the connected parts is unnecessary, but drying is desirable. This is conveniently done by sucking air through the apparatus.

It will be noted that the device has been designed to allow the units to be assembled in a compact form, thus they can be mounted rigidly on a single iron stand and the whole easily transported without danger of breakage.

Formyl-d,l-Valine.

59 gm. (0.5 mol) of dry *d,l*-valine, 138 gm. (3 mol) of anhydrous formic acid, and some boiling stones are placed in the 500 cc. Pyrex reaction flask. The separator is filled with pure toluene up to the level of its outflow. A small amount of toluene is introduced in the return tube. The flow of water through the condenser at the top of the column is adjusted to about 60 drops per minute. The flask is heated in an oil bath kept at 145°. When the mixture is at boiling temperature (117–118° inside) it is refluxed for 2 hours, the column acting as a reflux condenser. Then bath and

flask are covered with an asbestos sheeting in order to prevent excessive heat losses and the temperature of the bath is raised to about 175°, at the same time 20 cc. of toluene are introduced drop by drop from the graduated funnel into the reaction flask. At the moment the last of the toluene is introduced, the first drops of distillate should appear at the outlet of the spiral condenser. The heating of the bath is discontinued and the bath allowed to cool to 160° at which temperature it is maintained until the distillation equilibrium is established. The thermometer at the top of the column indicates 86° for a pressure of 760 mm. As the rate of the distillation tends to decrease, the temperature of the bath is raised again very gradually until the final peak of 175° is reached. The heterogeneous distillate drops in the toluene-filled separator. An equal volume of toluene is thus displaced and flows through the return tube into the reaction flask. The water-formic acid mixture settles as the heavier layer at the bottom of the separator. As already pointed out in the theoretical part, formation of two layers finally takes place in the reaction flask as the result of the replacement by toluene of the formic acid which is gradually removed. The condensation temperature of the vapor rises from 86° to about 88°, the pressure being 760 mm. Towards the end of the reaction, the rate of distillation decreases considerably. The reaction is almost complete when the volume of the water-formic acid mixture in the separator attains 95 cc. A small amount of formic acid still remains in the flask, for a mixture of 9 gm. of water (0.5 mol formed) and 115 gm. of formic acid (2.5 mol excess used) occupies a volume of 104 cc. at 25°. The time necessary for the distillation is approximately 2 hours. 80 cc. of toluene are then slowly introduced from the dropping funnel into the reaction flask while the bath is still kept at 175°. The flask is then disconnected and its hot contents quickly poured into a mortar, before the molten formyl derivative crystallizes. The mixture is well emulsified by stirring while the crystallization takes place and is then allowed to stand overnight in an empty desiccator. The crystals are filtered with suction at room temperature, thoroughly washed with toluene, and dried in a vacuum oven at 70°. Yield 70.9 gm. (97 per cent of the theory). The crude material thus obtained was already pure. It analyzed as follows:

5.015 mg. substance: 9.125 mg. CO_2 and 3.425 mg. H_2O.
0.1000 gm. " : 6.94 cc. 0.1 N HCl (Kjeldahl).
$C_6H_{11}O_3N$ (145.1). Calculated. C 49.62, H 7.64. N 9.65.
Found. " 49.61, " 7.64, " 9.71 (Kjeldahl).
" 0.04 (Van Slyke).

Moisture 0.59 per cent.

A sample was crystallized from 3 parts of hot water in the presence of some norit. The formyl-*d,l*-valine sintered at 144° and melted sharply at 145° (corrected). Fischer[5] reported sintering at 137° and melting between 139–144° (140–145° corrected).

Analysis: C 49.73, H 7.81, N 9.44 (Kjeldahl). Moisture 0.87 per cent.

Formyl-d,l-Leucine.

The preparation was carried out in a manner identical with that for formyl-*d,l*-valine. 65.6 gm. (0.5 mol) of *d,l*-leucine were used. The crude product was washed with toluene which had been slightly acidulated with anhydrous formic acid. It contained 0.4 per cent moisture and about 1.8 per cent untransformed *d,l*-leucine (0.16 per cent amino nitrogen by Van Slyke) and was therefore less pure than the corresponding product from *d,l*-valine. The yield was 77.6 gm. which is 95.4 per cent of the theory, after correction for the unchanged material.

For analysis a sample was crystallized from 3 parts of boiling water in the presence of some norit. The crystals were washed with water, then with anhydrous ether, and dried at 76° under diminished pressure. The formyl-*d,l*-leucine analyzed as follows:

3.765 mg. substance: 7.340 mg. CO_2 and 2.780 mg. H_2O.
5.815 " " : 0.461 cc. N (31°, 756 mm.).
$C_7H_{13}O_3N$ (159.11). Calculated. C 52.80, H 8.23, N 8.80.
Found. " 53.16, " 8.26, " 8.85.

It softened at 112° and melted between 115–116° (corrected). The same figures were found by Fischer and Warburg.[1]

Formyl-d,l-Phenylalanine.

The preparation was carried out in a manner identical with that for formyl-*d,l*-valine. 82.6 gm. (0.5 mol) of *d,l*-phenylalanine were used. The crude formyl derivative crystallized in

[5] Fischer, E., *Ber. chem. Ges.*, **39**, 2322 (1906).

the course of the operation. The contents of the reaction flask were poured into a mortar and the crystalline mass thoroughly crushed under the toluene. The suspension, after standing overnight in an empty desiccator, was filtered with suction. The crude reaction product was washed with toluene, then with anhydrous ether. It was finely powdered and dried in a vacuum oven at 70°. It contained 0.46 per cent moisture and about 2.6 per cent untransformed *d,l*-phenylalanine (0.19 per cent amino nitrogen by Van Slyke) and was therefore less pure than the corresponding product from *d,l*-valine. The yield was 94.9 gm. which is 95.3 per cent of the theory, after correction for the unchanged material.

This material was purified in the following way. It was shaken with 100 cc. of cold 1.0 N hydrochloric acid, then filtered with suction. The residue was washed with 150 cc. of water, then thoroughly with anhydrous ether, and dried. Weight, 87.4 gm. For analysis, a sample of this purified product was recrystallized twice from 11 parts of boiling 8 per cent (by weight) alcohol in the presence of some norit. The solutions were filtered while hot. The formyl compound was washed with water, then with anhydrous ether, and dried at 100° under diminished pressure.

5.180 mg. substance: 11.805 mg. CO_2 and 2.720 mg. H_2O.[6]
7.270 " " : 0.460 cc. N (33°, 758.5 mm.).
$C_{10}H_{11}O_3N$ (193.1). Calculated. C 62.15, H 5.74, N 7.25.
Found. " 62.14, " 5.87, " 7.04.

It softened slightly at 166.5° (corrected) and melted rather sharply at 168.0° ± 0.2° (corrected) with evolution of gas. Fischer and Schoeller[7] reported softening of their substance at 164° (165.5° corrected) and melting between 167–168° (168.8–169.8° corrected).

SUMMARY.

A method has been devised to permit the complete removal of the water formed in the reaction between formic acid and amino acids.

The water is removed from the reaction mixture by distillation in the presence of toluene which is continuously reintroduced into

[6] The author is indebted to Dr. A. Elek for carrying out the micro analyses reported in this work.

[7] Fischer, E., and Schoeller, W., *Ann. Chem*, **357**, 2 (1907).

the reaction flask of a suitable distilling device. The reaction thus proceeds almost to completion.

The formyl derivatives of *d,l*-valine, *d,l*-leucine, and *d,l*-phenylalanine were obtained in practically quantitative yield.

The author desires to express his thanks and appreciation to Dr. P. A. Levene for his encouragement and assistance in this work.

STUDIES ON RACEMIZATION.

IX. ACTION OF ALKALI ON KETOPIPERAZINES. ACTION OF HYDROCHLORIC ACID ON AMINO ACIDS, PEPTIDES, AND KETOPIPERAZINES.

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In previous communications of this series, it was pointed out that the ultimate object of the work was the comparison of respective rates of racemization of proteins, peptides, and of ketopiperazines in alkaline solutions as an aid for the interpretation of the chemical structure of proteins. Viewed from this angle the extents of racemization observed for various ketopiperazines may be regarded as being of the same order of magnitude although definite differences are evident from Table I. For the aliphatic series these differences are parallel with the differences in stability of the ketopiperazines, the rule being that *the extent of racemization increases with the stability of the ketopiperazine.* Hence, the extent of racemization is higher in systems unfavorable for hydrolysis, such as pyridine-water solutions. Naturally, both racemization and stability of a ketopiperazine are conditioned by the structure of the amino acid components. The influence of the proximity of the phenyl group to the asymmetric carbon atom is seen in the case of glycyl-levo-phenylglycine anhydride for which the observed extent of racemization was 100 per cent.

In previous racemization work on peptides and ketopiperazines the initial concentration of sodium hydroxide allowed to react on these compounds was 0.1 N. All the ketopiperazines, with the exception of glycyl-levo-tyrosine anhydride, prepared in this work, were found to be sparingly soluble in this standard solution. The difficulty which already had presented itself in a previous study

with ketopiperazines containing a tertiary carbon atom was again overcome by the use of pyridine as a solvent. It was found convenient to operate in a 57 per cent by volume pyridine-water mixture containing 1 mol of sodium hydroxide per mol of ketopiperazine, the initial concentration of alkali being 0.1 N. All specific data and details of technique are given in the experimental part.

The extents of racemization were obtained by comparing the rotation of the significant amino acid, $[\alpha_l]_D^{25}$, in the final acid hydrolysate of the anhydride (after treatment with alkali) with the corresponding one, $[\alpha_0]_D^{25}$, in the acid hydrolysate of a sample of the anhydride not subjected to the action of alkali, all other conditions being the same. The extent of racemization with alkali is given, in per cent, by the ratio

$$\frac{[\alpha_0]_D^{25} - [\alpha_l]_D^{25}}{[\alpha_0]_D^{25}} \times 100$$

The values obtained for various ketopiperazines studied so far are given in Tables I and II. In Table I these ketopiperazines are arranged in the order of their increasing ring stability.

The figures secured for glycyl-levo-tyrosine anhydride are given in Table II, the separate arrangement suggesting itself because of the different behavior of this anhydride. From hydrolysis experiments it appears that glycyl-levo-tyrosine anhydride is somewhat more stable than the corresponding phenylalanine compound and higher racemization figures than those found were expected. In fact, these figures are not comparable with the ones given in Table I. It was not possible to realize conditions identical or at least equivalent to those observed for the other ketopiperazines because of the disturbing presence of the hydroxyl group in the molecule. When the ratio of alkali to anhydride is increased to 2, conditions conducive to faster hydrolysis of the anhydride and consequently to lesser racemization are created, while with the ratio of 1.6 the amount of alkali available for further enolization is apparently not sufficient.

The opportunity may now be taken to emphasize conditions under which a racemization is produced by the action of hydrochloric acid. Partial racemization occurred when the ketopiperazines, containing the active amino acids, phenylglycine, phenyl-

alanine, and tyrosine, were hydrolyzed at 100° with 20.3 per cent hydrochloric acid. On the other hand, with glycyl-levo-aspara-

TABLE I.

Racemization of Various Ketopiperazines with Alkali.

Ketopiperazines, in order of increasing ring stability.*	Racemization,† after 48 hrs. with 0.1 N NaOH; 1 mol per mol of ketopiperazine.	
	In water.	In 57 per cent by volume pyridine-water.
	per cent	*per cent*
Glycyl-levo-alanine anhydride.....	67 (at 18°)‡	
Glycyl-levo-asparagine "		89
Glycyl-levo-phenylglycine "		100
Glycyl-levo-leucine "	73 (at 32°)§	
Glycyl-dextro-phenylalanine "		92
Glycyl-dextro-valine "	84 (at 32°)§	
Glycyl-dextro-isovaline anhydride. ¶.....	None (in 10 per cent by volume pyridine-water).§	

* Levene, P. A., Rothen, A., Steiger, R. E., and Osaki, M., *J. Biol. Chem.*, **86**, 723 (1930).

† Temperature 25°, unless otherwise stated.

‡ Levene, P. A., and Pfaltz, M. H., *J. Gen. Physiol.*, **8**, 185, 188 (1925).

§ Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 313, 317 (1928).

¶ Tertiary asymmetric carbon atom.

TABLE II.

Racemization of Glycyl-Levo-Tyrosine Anhydride with Alkali.

1 mol anhydride + x mols NaOH, in water, 48 hrs. at 25°.	Racemization.*
	per cent
With 1.0 mol of NaOH.....	10 (?)
" 1.6 " " "	∞ 62
" 2.0 " " "	64

* The values, particularly the first one, are somewhat uncertain (see Section 4 of the experimental part).

gine anhydride which contains an active amino acid of aliphatic nature and which is also rapidly hydrolyzed there was practically no racemization. Partial racemization also occurred in the case

of the peptides, glycyl-levo-phenylglycine and glycyl-dextro-phenylalanine, while there was little or none in the case of glycyl-levo-asparagine. The free amino acids behaved differently. A marked progressive loss of optical activity was observed when levo-phenylglycine was heated to 100° with 20.3 per cent hydrochloric acid (see Table IV), while there was no corresponding change in the case of dextro-phenylalanine.[1]

We wish to thank Dr. Masao Osaki and Mr. R. E. Marker for their valuable assistance in the preparation of the compounds used in these experiments.

EXPERIMENTAL.

1. Preparation of Compounds.

Glycyl-Levo-Asparagine Anhydride.

A. Levo-Asparagine.—The commercial hydrated product was used.

B. Chloroacetyl-Levo-Asparagine.—The directions of Fischer and Koenigs[2] were somewhat modified. The proportions of the reagents were the following. 75 gm. (0.5 mol) of levo-asparagine, dissolved in 500 cc. of 1.0 N sodium hydroxide (0.5 mol), 57 gm. (0.5 mol) of chloroacetyl chloride and 500 cc. of 1.0 N sodium hydroxide (0.5 mol), finally 100 cc. of 5.0 N hydrochloric acid (0.5 mol). The solution was concentrated to dryness under reduced pressure. The residue was thoroughly dried by reevaporating it twice in the presence of a mixture of absolute alcohol and benzene. The chloroacetyl compound was extracted with hot absolute alcohol. It crystallized on concentrating the filtrate from sodium chloride under reduced pressure. It was finally recrystallized from hot 95 per cent alcohol. It melted at 149–150° (uncorrected). Fischer and Koenigs reported for their material a melting point of 148–149° (corrected).

[1] Fischer, E., and Weichhold, O., *Ber. chem. Ges.*, **41**, 1287 (1908) observed complete racemization in the reaction of nitrous acid or nitrosyl-bromide and dextro-phenylglycine. It had been previously established that similar treatment of dextro-phenylalanine led to but little racemization.

[2] Fischer, E., and Koenigs, E., *Ber. chem. Ges.*, **37**, 4587 (1904).

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C. Glycyl-Levo-Asparagine.—41.7 gm. (0.2 mol) of chloroacetyl-levo-asparagine were allowed to stand 4 days at room temperature with 10 times this weight of ammonium hydroxide (sp. gr. 0.90). The solution was then diluted with water and concentrated to dryness under reduced pressure. The residue was dissolved in 100 gm. of water and the evaporation to dryness repeated. The mixture of dipeptide and of ammonium chloride was dissolved in 100 gm. of water, in the presence of some norit, and 170 cc. of absolute alcohol were added to the filtrate. At first, a slight oily precipitate was formed, it became crystalline on standing or cooling, then 200 cc. additional alcohol were added gradually under shaking. The mixture was allowed to stand overnight in the ice box. The crystals were washed with 60 per cent by weight alcohol, then with absolute alcohol. Yield, 32 gm. This product was dissolved in 150 gm. of water and norit was added; 180 cc. of hot absolute alcohol were added to the filtrate. Crystallization set in slowly, then 200 cc. additional alcohol were added gradually under shaking. The mixture was allowed to cool in the ice box. The crystals were washed with absolute alcohol. Yield, 28 gm. The substance was further purified. It was dissolved in 150 gm. of water and norit was added; 170 cc. of hot absolute alcohol were added to the filtrate and the solution allowed to cool in the ice box. Yield, 23.8 gm. For analysis the substance was dried at 76° under diminished pressure.

No. 178.

3.940 mg. substance: 5.520 mg. CO_2 and 2.100 mg. H_2O.
4.275 " " : 0.832 cc. N (26°, 762 mm.).
$C_6H_{11}O_4N_3$ (189.12). Calculated. C 38.07, H 5.86, N 22.22.
Found. " 38.20, " 5.96, " 22.28.
Moisture 0.37 per cent.

It showed the following rotation.

$$[\alpha]_D^{25} = \frac{-2.27° \times 100}{4 \times 7.565} = -7.50° \text{ (in water).}$$

1.1347 gm. (dry) in 15.00 cc. of solution (0.4 M).

A "glycyl-asparagine" had been previously obtained by Fischer and Koenigs,[2] on heating chloroacetyl-levo-asparagine with 25 per cent ammonium hydroxide at 100°. An approximate deter-

mination made on a 7 per cent aqueous solution gave $[\alpha]_D^{20} = -6.4°$.

D. Glycyl-Levo-Asparagine Anhydride.—A suspension of 19 gm. (0.1 mol) of glycyl-levo-asparagine in 190 cc. of dry methyl alcohol was saturated with dry hydrogen chloride gas while well cooled in ice. The solution was evaporated to dryness under reduced pressure and at a temperature not exceeding 30°. A second esterification identical to the first was then performed. The residue obtained on concentration was reevaporated twice with 25 cc. of benzene in order to remove more effectively the free hydrochloric acid. It was then dissolved in 50 cc. of cold dry methyl alcohol. To this solution, kept in ice, 140 cc. of methyl alcohol saturated with ammonia gas at 0° were added in one portion. Crystallization took place immediately. The mixture was saturated at 0° with dry ammonia gas and then allowed to stand 3 days in ice. The crystals were filtered off and were washed thoroughly with dry methyl alcohol. Yield, 11.8 gm. The crude anhydride was crystallized from 30 times its weight of hot 60 per cent by weight alcohol. For analysis, the substance was dried at 100° under diminished pressure.

No. 205.

5.065 mg. substance: 7.825 mg. CO_2 and 2.465 mg. H_2O.
5.350 " " : 1.171 cc. N (31°, 753.4 mm.).
$C_5H_9O_3N_3$ (171.1). Calculated. C 42.08, H 5.30, N 24.56.
Found. " 42.13, " 5.40, " 24.33.
Moisture 2.12 per cent.

It showed the following rotation.

$$[\alpha]_D^{25} = \frac{+ 3.20° \times 100}{4 \times 1.7114} = + 46.7° \pm 0.1° \text{ (in pyridine-water).}$$

0.2567 gm. (dry) + 10.0 cc. water + pyridine to make 15.00 cc. at 25° (0.1 M).

Glycyl-Levo-Phenylglycine Anhydride.

A. Levo-Phenylglycine.—The sample used for the preparation of the derivatives listed below and in a series of racemization experiments has been described briefly in a previous publication.[3]

[3] Levene, P. A., Steiger, R. E., and Bass, L. W., *J. Biol. Chem.*, **82**, 160 (1929).

Some additional information is given here. By mistake, the rotations published were reported as having been measured with sodium light D line, instead of Hg light of wave-length 5461 Å. and the concentrations given as 0.25 M instead of correctly 0.04 M. This purified material (dry) contained 5.5_7 per cent of dextro-phenylglycine. 9.70 cc. of 1.0 N hydrochloric acid and 4.34 cc. of water were added to 1.0744 gm. of dry substance and α_D^{25} was $-40.52°$ (in a 4 dm. tube). Fischer and Weichhold,[4] using identical conditions, found $\alpha_D^{20} = -22.80°$ (in a 2 dm. tube) with a highly pure material obtained on resolving formyl-*d,l*-phenylglycine. By means of a multiplication factor of 1.125_4 the following rotations for pure levo-phenylglycine are obtained,

$$[\alpha]^{25}_{Hg\ 5461} = -125.8° \text{ (in water)}$$

$$[\alpha]^{25}_{Hg\ 5461} = -164.9° \text{ (with 1 equivalent of HCl)}$$

$$[\alpha]^{25}_{Hg\ 5461} = -130.9° \text{ (with 1 equivalent of NaOH)}$$

the concentrations of all solutions being 0.04 M.

The impure levo-phenylglycine at our disposal showed a rotation of

$$[\alpha]^{25}_D = \frac{-8.86° \times 100}{4 \times 1.494} = -148.3° \text{ (in 16.5 per cent HCl).}$$

0.3777 gm. (dry) + 20.0 cc. of 20.3 per cent HCl + 5.0 cc. of water, volume of the solution: 25.28 cc. at 25°.

Since it contained 5.5_7 per cent of dextro isomer it follows that for pure levo-phenylglycine

$$[\alpha]^{25}_D = -166.9° \text{ (in 16.5 per cent HCl.)}$$

This value is in excellent agreement with Fischer and Weichhold's $[\alpha]^{20}_D = -165.43° \pm 0.4°$ (in 10 per cent HCl),[4] for as known, the value of the rotation of this amino acid increases with the increase in the concentration of the hydrochloric acid used as a solvent.[5] A slightly higher figure than Fischer and Weichhold's was expected.

[4] Fischer, E., and Weichhold, O., *Ber. chem. Ges.*, **41**, 1292 (1908).

[5] Levo-phenylglycine and dextro-phenylalanine are configurationally related. The rotation changes in each case in the same direction; *i.e.*, becomes more negative with the increase in concentration of the hydrochloric acid.

B. Chloroacetyl-Levo-Phenylglycine.—The preparation of this substance has already been described.[3]

C. Glycyl-Levo-Phenylglycine.—The preparation has been described in a previous publication.[3] Purified dipeptide, $[\alpha]_D^{25} = -173.9°$, was used for racemization experiments with acid and crude dipeptide for preparing the following anhydride.

D. Glycyl-Levo-Phenylglycine Anhydride.—A suspension of 11.2 gm. of the above dipeptide in 112 cc. of dry methyl alcohol was saturated with dry hydrogen chloride gas, the reaction flask being immersed in cold water. The solution was evaporated to dryness under reduced pressure. The esterification was then repeated. The residue left after evaporation was reevaporated twice with 25 cc. of benzene, in order to remove more effectively the free hydrochloric acid. It was then dissolved in 50 cc. of dry methyl alcohol, 112 cc. of methyl alcohol saturated with ammonia gas at 0° were added. A clear solution resulted in which crystals appeared very soon. This mixture was cooled in ice, saturated with dry ammonia gas, and then allowed to stand overnight. The crystals were washed with methyl alcohol and dried. They weighed 3 gm. The mother liquor yielded 4.5 gm. of a less pure material containing chlorine, when concentrated under reduced pressure. 7.5 gm. of impure anhydride thus obtained were crystallized from 30 times this weight of boiling water in the presence of some norit. The crystals deposited after cooling in ice were washed with water, finally with alcohol, and dried at 60° under diminished pressure. Yield, 3.2 gm.

No. 171.

5.115 mg. substance: 11.925 mg. CO_2 and 2.435 mg. H_2O.
0.1000 gm. " : 10.53 cc. 0.1 N HCl (Kjeldahl).
$C_{10}H_{10}O_2N_2$ (190.1). Calculated. C 63.13, H 5.30, N 14.74.
Found. " 63.57, " 5.32, " 14.74.
Moisture 0.36 per cent.

It showed the following rotation.

$$[\alpha]_D^{23} = \frac{-11.69° \times 100}{4 \times 3.802} = -76.9° \text{ (in glacial acetic acid).}$$

0.5703 gm. (dry) in 15.00 cc. of solution (0.2 M).

This material probably contained as much as 4.6 per cent of dextro isomer.

Glycyl-Dextro-Phenylalanine Anhydride.

A. Dextro-Phenylalanine.—*d,l*-Phenylalanine was formylated.[6] The formyl-*d,l*-phenylalanine was resolved by means of brucine according to the directions of Fischer and Schoeller.[7] The sample used for a series of racemization experiments showed the following rotation.

$$[\alpha]_D^{25} = \frac{+ 2.39_5° \times 100}{4 \times 1.7500} = + 34.21° \text{ (in water).}$$

0.2625 gm. (dry) in 15.00 cc. of solution.

Since Fischer and Schoeller found for pure dextro-phenylalanine $[\alpha]_D^{20} = +35.14°$ ($\pm 0.5°$), employing a solution of 0.1259 gm. in water, the weight of the solution being 7.2187 gm., the above sample contained 1.3 per cent of levo-phenylalanine. Its rotation in hydrochloric acid was then determined.

$$[\alpha]_D^{25} = \frac{+ 0.58_5° \times 100}{4 \times 2.0279} = + 7.21° \text{ (in 10.6 per cent HCl).}$$

0.5159 gm. (dry) + 12.5 cc. of 20.3 per cent HCl + 12.5 cc. of water; volume of the solution: 25.44 cc. at 25°.

It follows that for pure dextro-phenylalanine

$$[\alpha]_D^{25} = + 7.40° \pm 0.06° \text{ (in 10.6 per cent HCl).}$$

Fischer and Mouneyrat[8] found for pure dextro-phenylalanine $[\alpha]_D^{20} = +7.07°$ (in 18 per cent HCl). The value of the rotation of this amino acid decreases with the increase in concentration of the hydrochloric acid used as a solvent.[5]

B. Chloroacetyl-Dextro-Phenylalanine.—A solution of 33 gm. (0.2 mol) of dextro-phenylalanine in 100 cc. of 2.0 N sodium hydroxide (0.2 mol) was continually shaken in an ice-water mixture, and treated alternately with 23 gm. (0.2 mol) of chloroacetyl chloride and 120 cc. of 2.0 N sodium hydroxide (0.24 mol), the reagents being added in equivalent proportions. The chloroacetyl derivative precipitated on addition of 60 cc. of 5.0 N hydro-

[6] Steiger, R. E., *J. Biol. Chem.*, **86**, 700 (1930).
[7] Fischer, E., and Schoeller, W., *Ann. Chem.*, **357**, 1 (1907).
[8] Fischer, E., and Mouneyrat, A., *Ber. chem. Ges.*, **33**, 2386 (1900).

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chloric acid (0.3 mol). It was washed with ice water, then with petrolic ether and dried in a vacuum desiccator over phosphorus pentoxide and soda lime. It weighed 37.6 gm. (Yield, 78 per cent of the theory).

C. Glycyl-Dextro-Phenylalanine.—46.7 gm. of crude chloroacetyl-dextro-phenylalanine were allowed to stand 5 days at 37° with 10 times this weight of ammonium hydroxide (sp. gr. 0.90). The mixture was shaken occasionally in order to bring about a more rapid dissolution of the chloroacetyl compound. The solution was then diluted with an equal volume of water, shaken with norit, filtered, and concentrated to dryness under reduced pressure. The residue was twice reevaporated to dryness with 100 cc. of water, and then dissolved in hot water. The solution which weighed 880 gm. was treated with norit and filtered while hot. 1500 cc. of absolute alcohol were added to the filtrate. This mixture was left to cool overnight in ice. The dipeptide which had crystallized was filtered off, washed with 60 per cent by weight alcohol, and finally with absolute alcohol. It weighed 24 gm. It was dissolved in 20 parts of hot water in the presence of some norit. 1500 cc. of absolute alcohol were added to the filtrate. Crystallization of the dipeptide set in immediately. The mixture was then left to cool in ice for 2 days. The dipeptide which weighed 19.5 gm. was dissolved again in 20 parts of hot water in the presence of some norit. 1500 cc. of absolute alcohol were added to the filtrate. 16.5 gm. of dipeptide were obtained.

No. 211.

5.120 mg. substance: 11.220 mg. CO_2 and 2.895 mg. H_2O.
4.230 " " : 0.462 cc. N (27°, 758 mm.).
$C_{11}H_{14}O_3N_2$ (222.13). Calculated. C 59.43, H 6.35, N 12.61.
Found. " 59.75, " 6.32, " 12.40.
Moisture 1.43 per cent.

It showed the following rotations.

$$[\alpha]_D^{25} = \frac{-3.71_5° \times 100}{4 \times 2.2483} = -41.31° \text{ (in water)}.$$

0.3332 gm. (dry) in 14.82 cc. of solution (0.1 M).

$$[\alpha]_D^{25} = \frac{-3.16° \times 100}{4 \times 1.9096} = -41.37° \text{ (in water)}.$$

0.2874 gm. (dry) in 15.05 cc. of solution.

This peptide contained 0.8 per cent of levo isomer. Fischer and Schoeller[7] found for glycyl-levo-phenylalanine $[\alpha]_D^{20} = +41.4°$ ($\pm 0.5°$), employing a solution of 0.2523 gm. in water, the weight of the solution being 12.3340 gm. and on a recrystallized sample $[\alpha]_D^{20} = +42.0°$ ($\pm 0.5°$), employing a solution of 0.1350 gm. in water, the weight of the solution being 7.0758 gm.

D. Glycyl-Dextro-Phenylalanine Anhydride.—A suspension of 11.1 gm. of glycyl-dextro-phenylalanine in 111 cc. of dry methyl alcohol was saturated with dry hydrogen chloride gas, the reaction flask being immersed in cold water. The solution was evaporated to dryness under reduced pressure. The esterification was then repeated. The crystalline residue obtained on evaporation was reevaporated twice with 25 cc. of benzene in order to remove more effectively the free hydrochloric acid. It was then dissolved in 50 cc. of warm ethyl alcohol. To this solution 111 cc. of an ethyl alcoholic ammonia solution (saturated at 0°) were added in one portion. The mixture, kept standing in ice was saturated with dry ammonia gas. The anhydride precipitated as a gel. The mixture was allowed to stand 4 hours in the cold room, and then filtered with suction. The residue was washed with absolute alcohol, then with ether and air-dried. It was dissolved in 700 cc. of boiling water in the presence of some norit. The solution was filtered while hot. The anhydride separated as a gel. It crystallized when the mixture was allowed to cool overnight in ice. The anhydride was filtered, washed with water, then with alcohol and ether. It weighed 5.1 gm. On concentrating the filtrate an additional 1.1 gm. of anhydride were obtained. The combined fractions were dissolved in 672 cc. of boiling water in the presence of some norit. The filtrate was kept standing in ice for 2 days. The anhydride was filtered and washed with water and alcohol. Yield, 5.7 gm.

No. 215.

4.785 mg. substance: 11.355 mg. CO_2 and 2.480 mg. H_2O.
5.430 " " : 0.670 cc. N (29°, 760 mm.).
$C_{11}H_{12}O_2N_2$ (204.12). Calculated. C 64.67, H 5.93, N 13.72.
Found. " 64.71, " 5.79, " 13.95.
Moisture 0.48 per cent.

It showed the following rotation.

$$[\alpha]_D^{25} = \frac{-8.23° \times 100}{4 \times 2.0420} = -100.76° \text{ (in glacial acetic acid).}$$

0.3063 gm. (dry) in 15.00 cc. of solution (0.1 M).

Fischer and Schoeller[7] found for glycyl-levo-phenylalanine anhydride $[\alpha]_D^{20} = +100.5°$ ($\pm 0.4°$), employing a solution of 0.2521 gm. in glacial acetic acid, the weight of the solution being 9.8232 gm. and on a recrystallized sample $[\alpha]_D^{20} = +99.5°$ ($\pm 0.5°$), employing a solution of 0.1391 gm. in glacial acetic acid, the weight of the solution being 6.6871 gm.

Starting from the amino acid, an entirely independent preparation of the anhydride was made. The analysis was the following.

No. 210.
5.100 mg. substance: 12.070 mg. CO_2 and 2.615 mg. H_2O.
5.350 " " : 0.656 cc. N (29°, 758 mm.).
$C_{11}H_{12}O_2N_2$ (204.12). Calculated. C 64.67, H 5.93, N 13.72.
Found. " 64.53, " 5.73, " 13.82.
Moisture 0.55 per cent.

and the rotation was the same as that above.

$$[\alpha]_D^{23} = \frac{-8.24° \times 100}{4 \times 2.0448} = -100.74° \text{ (in glacial acetic acid).}$$

0.3063 gm. (dry) in 14.98 cc. of solution (0.1 M).

Glycyl-Levo-Tyrosine Anhydride.

Levo-tyrosine (obtained from silk) was converted into its ethyl ester hydrochloride. The chloroacetyl-levo-tyrosine ethyl ester prepared according to Fischer[9] was treated with ethyl alcoholic ammonia.[10] The first fraction, after purification, analyzed as follows:

No. 172.
5.065 mg. substance: 11.145 mg. CO_2 and 2.510 mg. H_2O.
0.1000 gm. " : 9.05 cc. 0.1 N HCl (Kjeldahl).
$C_{11}H_{12}O_3N_2$ (220.12). Calculated. C 59.97, H 5.50, N 12.73.
Found. " 60.00, " 5.55, " 12.67.
Moisture 0.39 per cent.

For comparison, the rotation was measured in an ammonium hydroxide solution of the same concentration as that used by Fischer and Schrauth. Prolonged shaking and finally warming

[9] Fischer, E., *Ber. chem. Ges.*, **37**, 2494 (1904).
[10] Fischer, E., and Schrauth, W., *Ann. Chem.*, **354**, 28, (1907).

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up of the suspension was necessary to bring about complete dissolution; the reading was taken immediately thereafter.

$$[\alpha]_D^{25} = \frac{+ 9.96° \times 100}{4 \times 2.0578} = + 121.0°.$$

0.3086 gm. (dry) + 7 cc. of water + 7.5 cc. of ammonium hydroxide (sp. gr. 0.90) + water to make 15.00 cc. at 25°.

Fischer and Schrauth[10] found varying values $[\alpha]_D^{20} = +125.4°$, $+126.4°$, $+124.3°$, employing solutions of 0.1879 gm. in weak aqueous ammonium hydroxide, the weight of the solutions being 8.5150 gm. and the density 0.9541.

Another sample of glycyl-levo-tyrosine anhydride, isolated from the mother liquors of the preparation of the preceding, and purified separately, analyzed as follows:

No. 176.
5.180 mg. substance: 11.365 mg. CO_2 and 2.500 mg. H_2O.
0.1000 gm. " : 9.03 cc. 0.1 N HCl (Kjeldahl).
$C_{11}H_{12}O_3N_2$ (220.12). Calculated. C 59.97, H 5.50, N 12.73.
Found. " 59.83, " 5.40, " 12.64.
Moisture 0.06 per cent.

and showed a rotation of

$$[\alpha]_D^{25} = \frac{+ 9.94° \times 100}{4 \times 2.0546} = + 120.9°.$$

0.3086 gm. (dry) + 7 cc. of water + 7.5 cc. of ammonium hydroxide (sp. gr. 0.90) + water to make 15.02 cc. at 25°.

2. Racemization of Ketopiperazines in Alkaline Solutions at 25°.

Alkaline solutions of the four ketopiperazines prepared were allowed to stand 48 hours in a thermostat kept at 25°. All the specific data are recorded in Table III.

The solutions were quantitatively transferred into 100 cc. flasks and neutralized for the amount of NaOH (or NaOH and NH_3 in the case of the derivative of asparagine) present. They were then concentrated to dryness under reduced pressure. For this purpose, 50 cc. Pyrex test-tubes (180 × 20 mm.) were used, equipped with a wide distilling head carrying a dropping funnel and a capillary reaching to the bottom of the tube. The solu-

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TABLE III.

Racemization of Ketopiperazines in Alkaline Solutions at 25°.

Ketopiperazine	Glycyl-levo-asparagine anhydride, No. 205.	Glycyl-levo-phenylglycine anhydride, No. 171.	Glycyl-dextro-phenyl-alanine anhydride, No. 215.	Glycyl-levo-tyrosine anhydride, No. 172.		
Amount of substance (0.0025 mol, corrected for moisture), *gm.*	0.4370	0.4770	0.5128	0.5525		
Solvent-reagent.	14.25 cc. pyridine, 7.5 cc. water* + 2.50 cc. 1.0 N NaOH (1.0 mol).			22 cc. water,* 2.5 cc. 1.0 N NaOH (1.0 mol).	20.2 cc. water,* 4.0 cc. 1.0 N NaOH (1.6 mol).	19.2 cc. water,* 5.0 cc. 1.0 N NaOH (2.0 mol).
	then water quickly to bring volumes of solutions to 25.0 cc. at 25°.					
Amount of 1.0 N HCl used for neutralizing solutions after 48 hrs., *cc.*	5.0	2.5	2.5	2.5	4.0	5.0
Amount of 20.3 per cent HCl used for hydrolysis, *cc.*	20.0	20.0	10.0	10.0 (and 0.2 gm. norit).		
Amount of water used for diluting the hydrolysate, *cc.*	None.	5.0	10.0	10.0 (solutions filtered from norit; still colored).		
Total N in the diluted hydrolysate, *gm. per 100 cc.*	I 0.5270 II 0.5425	0.2715	0.3330	0.3255		
α_{1D}^{25} (4 dm. tube).	I +0.17° II +0.18$_5$°	0.00°	+0.04°	−0.59°	−0.25°† (or −0.26°?)	−0.23$_5$°† (or −0.24°?)

$[\alpha_l]_D^{25}$ of the significant amino acid in solution.	I $+2.5_2°$ II $+2.6_2°$	0.00°	$+0.5_1°$	$-7.0_2°$	$-2.9_7°$ (or $-3.0_9°$?)	$-2.8°$ (or $-2.8_5°$?)
$[\alpha_6]_D^{25}$ of the significant amino acid in solution, after direct hydrolysis of the anhydride (from Table V).	$+24.1°$	$-124.8°$	$+6.0_7°$			$-7.8_2°$

* Up to this point, there was complete solution only in the case of glycyl-levo-phenylglycine anhydride; in the other cases, it was brought about by the addition with shaking of the alkali or upon dilution to 25.0 cc. The suspensions of glycyl-levo-tyrosine anhydride were thoroughly cooled in ice water, before the alkali was added.

† Reading difficult due to color of solutions.

tions were introduced gradually through the dropping funnel. In order to remove the pyridine entirely, the evaporation was repeated several times after addition each time of water. The dry residues, consisting of anhydrides, their split-products, and sodium chloride, were taken up in concentrated hydrochloric acid. The acid was added from a pipette through the distilling heads to wash down some dry material that had collected there and on the upper part of the sides of the tubes. The test-tubes were then sealed and immersed for 36 hours in a steam-heated water bath at 100°. After hydrolysis, an amount of water sufficient to prevent crystallization of the hydrochlorides of the racemic amino acids was added to each solution. Total nitrogen (Kjeldahl), amino nitrogen (Van Slyke), and ammonia nitrogen (in the case of the derivative of asparagine) were determined and the concentrations of the

TABLE IV.

Racemization of Levo-Phenylglycine with 20.3 Per Cent HCl at 100°.

Time.	α_D^{25}*	$[\alpha]_D^{25}$*	Loss of optical activity.	
hrs.	*degrees*	*degrees*	*in degrees*	*in per cent*
0		−148.3		
6	−8.60	−142.6	5.7	3.8₄
24	−7.80	−129.3	19.0	12.8
36	−7.36	−122.0	26.3	17.7₃

* In 16.5 per cent HCl.

significant amino acids (aspartic acid, phenylglycine, phenylalanine, tyrosine) calculated. The rotations of the solutions were measured and the specific rotations, $[\alpha_1]_D^{25}$, of the significant amino acids, calculated and compared with the values, $[\alpha_0]_D^{25}$, similarly obtained on direct acid hydrolysis of the anhydrides, the treatment with alkali having been omitted.

3. Racemization of Amino Acids in Hydrochloric Acid Solutions at 100°.

I. Levo-Phenylglycine.—0.0025 mol (corrected for moisture) of levo-phenylglycine containing 5.5₇ per cent of dextro isomer, were heated for varying lengths of time with 20.0 cc. of 20.3 per cent hydrochloric acid, in sealed tubes immersed in a steam-

heated water bath at 100°. The solutions in each case were diluted with 5.0 cc. of water, their nitrogen content (5.59 mg. in 4 cc., in every case) determined, and their rotations measured in 4 dm. tubes. The figures obtained are given in Table IV. The values of $[\alpha]_D^{25}$ lie on a smooth curve.

II. Dextro-Phenylalanine.—0.0025 mol (corrected for moisture) of dextro-phenylalanine, containing 1.3 per cent of levo isomer, were heated for 12, 24, and 36 hours with 10.0 cc. of 20.3 per cent hydrochloric acid in sealed tubes at 100°. The solutions were diluted with 10.0 cc. of water, their nitrogen content determined, and their rotations, α_D^{25}, measured in 4 dm. tubes. The calculated specific rotations, $[\alpha]_D^{25}$ (duly corrected for the amount of levo isomer present), were in each case well within $+7.40° \pm 0.06°$ which is the rotation of the blank. Hence, there was no racemization of dextro-phenylalanine under the conditions used.

4. Racemization of Dipeptides and Ketopiperazines in Hydrochloric Acid Solutions at 100°.

Various dipeptides and ketopiperazines were hydrolyzed with 20.3 per cent hydrochloric acid in sealed tubes immersed for 36 hours in a steam-heated water bath at 100°. All the experimental data are recorded in Table V.

The extents of racemization calculated, given below, do not necessarily represent the correct maximal values, for it is very likely that the peptides and anhydrides had suffered already a partial hydrolysis in their acid solutions at room temperature, that is, prior to their immersion in the bath at 100°. It is obvious that since, for instance, free dextro-phenylalanine suffers no racemization, while the reverse is true when this amino acid is linked up in peptide or anhydride forms, any such hydrolysis will considerably alter the results. In fact, in a series of experiments carried out on precisely these compounds great discrepancies were observed, thus making apparent the need of special precautions. It was then, however, too late to repeat all these experiments. Therefore, in any comparisons of the following figures (and in the interpretation of the racemization findings on glycyl-levo-tyrosine anhydride with alkali) this point has to be well kept in mind.

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TABLE V.

Racemization of Dipeptides and Ketopiperazines in Hydrochloric Acid Solutions at 100°.

Compound	Glycyl-levo-asparagine, No. 178.	Glycyl-levo-asparagine anhydride, No. 205.	Glycyl-levo-phenyl-glycine, No. 167.	Glycyl-levo-phenyl-glycine anhydride, No. 171.	Glycyl-dextro-phenylalanine, No. 211.	Glycyl-dextro-phenyl-alanine anhydride, No. 215.	Glycyl-levo-tyrosine anhydride, No. 172.
Amount of substance (0.0025 mol, corrected for moisture), *gm.*	0.4746	0.4370	0.5251	0.4770	0.5634	0.5128	0.5525
Amount of NaCl added, *gm.*	0.1462	0.1462	0.1462	0.1462	0.1462	0.1462	0.2923
" " " " , *mol.*	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0050
Amount of 20.3 per cent HCl used for hydrolysis, *cc.*	20.0	20.0	20.0	20.0	10.0	10.0	10.0 (and 0.2 gm. norit).
Amount of water used for diluting the hydrolysate, *cc.*	None.	None.	5.0	5.0	10.0	10.0	10.0 (solution filtered).
Total N in the diluted hydrolysate, *gm. per 100 cc.*	0.5005 (NH_3 nitrogen = 0.1735, corrected).	0.5145 (NH_3 nitrogen = 0.1715).	0.2735	0.2748	I 0.3410 II 0.3430	0.3380 0.3430	0.3293
α_{5893}^{25} (4 dm. tube).	+1.50°	+1.57°	−8.12°	−7.40°	I +0.59° II +0.49$_5$°	+0.52° +0.49°	−0.66$_5$°
$[\alpha_5]_D^{25}$ of the significant amino acid in solution.	+24.1$_5$°	+24.1°	−137.6°	−124.8°	I +7.3$_5$° II +6.1$_2$°	+6.5$_3$° +6.0$_7$°	−7.8$_2$°
$[\alpha]_D^{25}$ of the significant amino acid in HCl (without glycine and NaCl).	I +24.2°, II +24.4° (in 20.3 per cent HCl).*		−166.9° (in 16.5 per cent HCl).		+7.40°±0.06° (in 10.6 per cent HCl).		Not determined.

* Two different samples of purified levo-aspartic acids were used. Their solutions contained equivalent amounts of glycine, NaCl, and NH_4Cl.

A comparison of the values $[\alpha_0]_D^{25}$ and $[\alpha]_D^{25}$ given in Table V shows that: Practically no racemization took place in the case of glycyl-levo-asparagine and its anhydride. The racemization of levo-phenyl-glycine in the peptide was 17.6 per cent (found: 17.7 per cent for free levo-phenylglycine, last figure in Table IV) and in the anhydride 25.2 per cent, if the peptide and anhydride used were optically pure. For the latter this may be questioned, since for the sake of conservation of material, the crude anhydride was recrystallized but once. An initial amount of 4.6 per cent of dextro isomer in the anhydride would account for the difference. The racemization of dextro-phenylalanine in the peptide was zero in Experiment I, while it was about 16 per cent in Experiment II (both $[\alpha_0]_D^{25}$ values given in Table V were corrected for 0.8 per cent of levo isomer originally present in the peptide used). These observations led to the considerations expressed above. Similar discrepancies were observed with the corresponding anhydride. The racemization was about 12 per cent in Experiment I, while in Experiment II, it was 18 per cent; hence, somewhat higher than that observed for the peptide. Finally, there was also partial racemization of levo-tyrosine in glycyl-levo-tyrosine anhydride. This results from the following considerations. Fischer[11] found for pure levo-tyrosine $[\alpha]_D^{20} = -8.64°$ (in 21 per cent hydrochloric acid) and $[\alpha]_D^{20} = -13.2°$ (in 4 per cent hydrochloric acid).[12] Value $[\alpha]_D^{25}$, for pure levo-tyrosine in 10.6 per cent hydrochloric acid (not determined), must lie somewhere between these extreme values. On the other hand, value $[\alpha_0]_D^{25}$ found after hydrolysis (see Table V) was $-7.8_2°$, or roughly corrected, is $-8.1_5°$, if the anhydride used really contained around 2 per cent of dextro isomer (see preparation). Our interest in this matter did not extend further.

Somewhat related observations made by Fischer may be mentioned here. Fischer[11] found that when benzoyl-levo-tyrosine

[11] Fischer, E., *Ber. chem. Ges.*, **32**, 3643, 3644 (1899).

[12] The value of the rotation of this amino acid decreases with the increase in concentration of the hydrochloric acid used as a solvent. Levo-tyrosine is configurationally related to levo-phenylalanine and dextro-phenylglycine. The rotation changes in each case in the same direction; *i.e.*, becomes more positive with the increase in concentration of the hydrochloric acid (*cf.* foot-note 5).

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was heated at 100° with 20 times its amount of hydrochloric acid of sp. gr. 1.19, almost half of the tyrosine obtained was inactive. Partial racemization took place also but to a much less extent when 20 per cent hydrochloric acid was used for hydrolysis. $[\alpha]_D^{20}$ for the isolated tyrosine was −7.4° (in 21 per cent hydrochloric acid), while $[\alpha]_D^{20}$ for pure levo-tyrosine was −8.64° (in 21 per cent hydrochloric acid). Hence, the racemization in this case was 14.4 per cent.

THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF KETOPIPERAZINES.

II. HYDROLYSIS OF KETOPIPERAZINES BY ALKALI.

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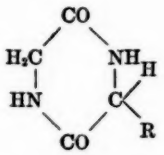
The grounds for this series of investigations have been discussed in previous communications and particularly in one by Levene, Bass, and Steiger.[1] It was pointed out that further information regarding the stability of ketopiperazines was required for the interpretation of the findings on the racemization of proteins.

In the present investigation observations were made on the behavior of a series of seven ketopiperazines, each of which contained glycine as one component. All were unsymmetric and were, but with one exception (namely, glycyl-dextro-isovaline anhydride), of the type



The rates of hydrolysis of these ketopiperazines were measured at 25° in sodium hydroxide solutions of an initial concentration of 0.5 N, 5 mols of sodium hydroxide per mol of ketopiperazine being used. It must be borne in mind that the average rate of hydrolysis of an unsymmetric ketopiperazine represents the resultant of the

[1] Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **81**, 697 (1929).

rates of formation of the two possible isomeric dipeptides; usually one isomer predominates in the mixture.

All the data are recorded in Tables II and III, while Fig. 1 gives the hydrolysis curves. In the following series the ketopiperazines are arranged in the order of increasing stability; glycyl-dextro-alanine anhydride ($k \cdot 10^3 = 95._7$), glycyl-levo-asparagine anhydride, glycyl-levo-phenylglycine anhydride ($k \cdot 10^3 = 24._7$), glycyl-levo-leucine anhydride ($k \cdot 10^3 = 20._8$), glycyl-levo-tyrosine anhydride ($k \cdot 10^3 = 5.3$?), glycyl-*d,l*-valine anhydride ($k \cdot 10^3 = 1.9_1$), glycyl-dextro-isovaline anhydride ($k \cdot 10^3 = 0.38$).

No hydrolysis constant was calculated for glycyl-levo-asparagine anhydride, a secondary hydrolysis, namely that of the amide, having been observed. Samples withdrawn at the end of the hydrolysis gave strong ammonia reactions with Nessler's reagent. This explains the high hydrolysis values found with the titration method. It will be seen from Table II that, for instance, the last value recorded there (3.05 cc.), exceeds by 22 per cent the theoretical value (2.50 cc.). The peptide linkage remained intact in glycyl-levo-asparagine when this peptide was submitted to hydrolysis under the same conditions as the anhydride; also, no liberation of ammonia was observed during the short time necessary to bring about the complete hydrolysis of the anhydride. It seems, therefore, that the amide is particularly unstable in the unchanged anhydride (in its keto or enol forms) and possibly also in levo-asparaginyl-glycine, the other possible primary hydrolysis product of the anhydride. This latter peptide was not available for experiments.

The hydrolysis of the sparingly soluble glycyl-dextro-phenylalanine anhydride was carried out in an alkaline solution containing a minimum amount of pyridine, the concentration and the proportion of sodium hydroxide being, however, the same as in the case of the other ketopiperazines. $k' \cdot 10^3$ was found to be 5.5_4. A set of parallel experiments performed on glycyl-levo-leucine anhydride in the absence and presence of pyridine, all other conditions being identical, show that pyridine has a great retarding influence on the hydrolysis. By estimation (based on the decrease of $k \cdot 10^3 = 20._8$ to $k' \cdot 10^3 = 10._7$ for the leucine compound) glycyl-dextro-phenylalanine anhydride appears to be less stable than glycyl-levo-tyrosine anhydride (due allowance being made in the

case of the latter anhydride for the lowering of the pH by the phenolic hydroxyl).

Thus, of the ketopiperazines composed of amino acids occurring in nature, studied in this work, glycyl-valine anhydride was found to possess the greatest stability, but even this ketopiperazine hydrolyzes to the extent of 89 per cent in the course of 8 hours. The anhydrides of amino acids as well as the mixed ketopiperazines not containing glycine have not yet been studied quantitatively, but the qualitative results of Fischer make it evident that they possess greater stability than the ones we have studied. Ketopiperazines in which the amino group is attached to a tertiary carbon atom (*e.g.*, glycyl-dextro-isovaline anhydride) are also quite resistant to the action of alkali. They are, however, of significance only for theoretical considerations inasmuch as tertiary amino acids are not known to occur in proteins. The work is being continued.

EXPERIMENTAL.

Preparation of Ketopiperazines.

The analyses of the ketopiperazines used in these experiments are summarized in Table I.

Glycyl-Dextro-Alanine Anhydride.—It was obtained from the hydrochloride of dextro-alanyl chloride[2] and glycine ethyl ester.[2] Cyclization took place when the hydrochloride of dextro-alanyl-glycine ethyl ester was neutralized. The anhydride was purified by crystallization from absolute alcohol. It had the following rotation.

$$[\alpha]_D^{25} = \frac{-0.34° \times 100}{2 \times 5.032} = -3.4° \text{ (in water).}$$

0.2516 gm. (dry) in 5.0 cc. of solution.

Partial racemization took place in the process of preparation since Fischer and Schulze[3] found $[\alpha]_D^{20} = -5.0°$ for the anhydride prepared from glycyl-dextro-alanine ethyl ester.

[2] Fischer, E., *Ber. chem. Ges.*, **38**, 2917, 2921 (1905).
[3] Fischer, E., and Schulze, A., *Ber. chem. Ges.*, **40**, 949 (1907).

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Glycyl-Levo-Asparagine Anhydride.—The preparation of this substance has been described in a preceding publication.[4]

Glycyl-d,l-Valine Anhydride.—This substance was prepared by fusing glycyl-*d,l*-valine at 250–260°. It was purified by repeated crystallization from absolute alcohol. This compound had been previously obtained from *d,l*-valyl-glycine by the same method.[5]

Glycyl-Dextro-Isovaline Anhydride.—The preparation of this substance was described previously.[6]

Glycyl-Levo-Leucine Anhydride.—This substance was prepared from glycyl-levo-leucine by Fischer's method[7] and crystallized from absolute alcohol.

$$[\alpha]_D^{25} = \frac{+\ 0.97^\circ \times 100}{2 \times 1.702} = +\ 28.5^\circ \text{ (in water).}$$

0.1702 gm. (dry) in 10.0 cc. of solution (0.1 M).

It must therefore have contained 4.2 per cent of glycyl-dextro-leucine anhydride since Fischer and Steingroever[8] found $[\alpha]_D^{20} = +31.1^\circ$ ($\pm$ 0.5°), employing a solution of 0.2131 gm. in water, the weight of the solution being 13.5161 gm.

Glycyl-Levo-Phenylglycine Anhydride.—The preparation of this substance has been described in a preceding publication.[4]

Glycyl-Dextro-Phenylalanine Anhydride.—Sample 210 of this substance, the preparation of which has been described in a preceding publication,[4] was used.

Glycyl-Levo-Tyrosine Anhydride.—Sample 176 of this substance, described in a preceding publication,[4] was used.

Procedure in Hydrolysis Experiments.

The following procedure was used for the ketopiperazines which were soluble in water or in the standard alkali required for the experiments. A modification of this procedure was necessary only in the case of glycyl-dextro-phenylalanine anhydride, which is separately discussed. The rates of hydrolysis were measured at

[4] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **86**, 703 (1930).
[5] Fischer, E., and Schenkel, J., *Ann. Chem.*, **354**, 15 (1907).
[6] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 306 (1928).
[7] Fischer, E., *Ber. chem. Ges.*, **39**, 2913 (1906).
[8] Fischer, E., and Steingroever, J., *Ann. Chem.*, **365**, 170 (1909).

25°, the initial concentration of sodium hydroxide acting on the ketopiperazines being 0.5 N. These conditions were the same as those adopted in a previous work.[1]

A sample of 0.0025 mol (corrected for moisture) of ketopiperazine was weighed in a 25.0 cc. volumetric flask. The sample was dissolved in water and 12.5 cc. of 1.0 N NaOH (5 mols per mol of ketopiperazine). The volume was made up to 25.0 cc. with water and the solution placed in a thermostat at 25°. At definite time intervals 2.50 cc. samples were withdrawn by means of pipettes; these samples were emptied into neutralizing solutions as described below.

TABLE I.

Analyses of Ketopiperazines.

Ketopiperazines.	Sample No.	Calculated.			Found.			
		C	H	N	C	H	N	Moisture.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Glycyl-dextro-alanine anhydride.	177	46.85	6.29	21.88	46.81	6.01	21.91	1.10
Glycyl-levo-asparagine anhydride	205	42.08	5.30	24.56	42.13	5.40	24.33	2.12
Glycyl-*d,l*-valine anhydride	183	53.81	7.75	17.95	54.02	7.35	17.71	0
Glycyl-dextro-isovaline anhydride	98	53.81	7.75	17.95	54.04	7.85	18.04	1.04
Glycyl-levo-leucine anhydride	199	56.43	8.29	16.47	56.58	7.92	16.40	0.65
Glycyl-levo-phenylglycine anhydride	171	63.13	5.30	14.74	63.57	5.32	14.74	0.36
Glycyl-dextro-phenylalanine anhydride	210	64.67	5.93	13.72	64.53	5.73	13.82	0.55
Glycyl-levo-tyrosine anhydride	176	59.97	5.50	12.73	59.83	5.40	12.64	0.06

The extent of hydrolysis was determined by titration in alcoholic solution in the presence of alizarin yellow and where this method was ineffective (glycyl-levo-tyrosine anhydride), by measuring the amino nitrogen in Van Slyke's apparatus.

Before beginning an experiment (on glycyl-dextro-alanine anhydride, glycyl-levo-asparagine anhydride, glycyl-*d,l*-valine anhydride, glycyl-dextro-isovaline anhydride, glycyl-levo-leucine anhydride, and glycyl-levo-phenylglycine anhydride), a series of neutralizing solutions was prepared, each solution consisting of 5 cc.

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TABLE II.—*Rates of Hydrolysis of Ketopiperazine*

Glycyl-dextro-alanine anhydride.			Glycyl-levo-asparagine anhydride.			Glycyl-*d*,*l*-valine anhydride.			Glycyl-isovaline anhydride.		
t	*V*	$k \cdot 10^3$	*t*	*V*	$k \cdot 10^3$	*t*	*V*	$k \cdot 10^3$	*t*	*V*	$k \cdot 10^3$
min.	*cc.*		*min.*	*cc.*		*min.*	*cc.*		*min.*	*cc.*	
2	0.97_5	(107)	2	0.80		5	0.06	(2.11)	211	0.37	(0.33)
3	1.37	(115)	2.75	1.30		74	0.74	(2.06)	339	0.74	(0.45)
4	1.56	(106)	5.25	1.85		83	0.78	1.96	501	0.91	0.39
5	1.70	99	7.33	2.15		178	1.38	1.96	960	1.30	(0.33)
7.08	1.97_5	95.7	9.02	2.26		258	1.69	1.90	1231	1.59	0.36
9.25	2.16	93.7	12.1	2.40		374	1.98	1.82	1479	1.84	0.39
11	2.27	94.2	18	2.68		471	2.19	1.92	1492	1.81	0.38
14	2.34		22.87	2.86		1365	2.47		2411	2.33	
47	2.48		26.75	2.92		Theory.	2.50		2791	2.38	
Theory.	2.50		48.5	3.05					Theor	2.50	
			Theory.	2.50							
Average.....		$95._7$			Not calculated.			1.9_1			0.38

* Values of $k \cdot 10^3$ in parentheses have not been used in calculating average cons. $k \cdot 10^3$ is cal

*lysis of piperazines by Sodium Hydroxide.**

Glycyl-isovaline anhydride.			Glycyl-levo-leucine anhydride.			Glycyl-levo-phenylglycine anhydride.			Glycyl-levo-tyrosine anhydride.		
t	*V*	$k \cdot 10^3$	*t*	*V*	$k \cdot 10^3$	*t*	*V*	$k \cdot 10^3$	*t*	*N*	$k \cdot 10^3$
min.	*cc.*		*min.*	*cc.*		*min.*	*cc.*		*min.*	*mg.*	
211	0.37	(0.33)	7.1	0.78	(22.9)	3	0.77		3	0.0316	
339	0.74	(0.45)	12.75	1.17	21.5	7.5	0.99	(29.2)	15.5	0.201	(4.3_4)
501	0.91	0.39	17.5	1.51	(23.0)	13	1.34	25.7	20.25	0.312	5.4
960	1.30	(0.33)	23	1.69	21.3	16.5	1.55	25.5	28.28	0.391	5.0_3
1231	1.59	0.36	28.75	1.91	21.8	20	1.68	24.2	36.50	0.495	5.2
1479	1.84	0.39	34	1.97	19.8	25	1.88	24.2	52.07	0.613	(4.8)
1492	1.81	0.38	51	2.25	19.6	30.5	2.05	24.4	72.5	0.814	5.2
2411	2.33		93.5	2.50		40	2.23	24.2	100	0.978	5.2
2791	2.38		Theory.	2.50		80	2.52		111.5	1.06	5.5
Theor	2.50					Theory.	2.50		124	1.11	5.5
									151	1.19	
									Theory.	1.40	
		0.38			$20._8$			$24._7$			5.3

cons. $k \cdot 10^3$ is calculated for minutes.

of alcohol, 1.25 cc. of 1.0 N hydrochloric acid and 2 drops of a 1 per cent solution of alizarin yellow. As soon as a sample had been removed from the reaction flask, it was emptied into one of these solutions. A solution of 0.1 N sodium hydroxide was then added from a small bore burette to the first definite red color. The samples were allowed to run into the neutralizing solutions at such a rate as to half empty the pipettes at the time intervals shown in the data.

TABLE III.

*Rates of Hydrolysis of Ketopiperazines by Sodium Hydroxide in Presence of Pyridine.**

Glycyl-dextro-phenylalanine anhydride.			Glycyl-levo-leucine anhydride.		
t	V	$k' \cdot 10^3$	t	V	$k' \cdot 10^3$
min.	*cc.*		*min.*	*cc.*	
9.25	0.34	(6.9)	3.25	0.31	(17.7)
20.25	0.60	5.9	8.50	0.58	(13.5)
28.75	0.83	6.1	15.28	0.92	(13.0)
46.07	1.04	5.1	26	1.18	10.7
63.5	1.39	5.5	43.53	1.68	11.1
90	1.66	5.3	59.5	1.88	10.2
129	1.98	5.3	77.5	2.14	10.9
182.25	2.25	5.5	125.5	2.44	
308	2.50		182	2.51	
Theory.	2.50		Theory.	2.50	
Average.....		5.5_4			$10._7$

* Values of $k' \cdot 10^3$ in parentheses have not been used in calculating average constants. $k' \cdot 10^3$ are calculated for minutes.

Before beginning an experiment (on glycyl-levo-tyrosine anhydride), a series of 5.0 cc. volumetric flasks each containing 2.5 cc. of glacial acetic acid was prepared. At definite time intervals 2.50 cc. samples were emptied into these flasks, the contents of which were then brought to the graduation mark with water.[9] The amino nitrogen determinations were made on 2.0_0 cc. of these mixtures. To the observed nitrogen values were applied the corrections obtained from the corresponding blanks. The results were expressed in mg. of nitrogen.

[9] The unchanged glycyl-levo-tyrosine anhydride remained in solution.

Because of the low solubility of glycyl-dextro-phenylalanine anhydride, it was necessary to modify the hydrolysis procedure employed for the other ketopiperazines. By replacing part of the water by pyridine, it was possible to bring this compound into solution without changing the concentration of alkali and the ratio of alkali to anhydride. A sample of 0.0025 mol (corrected for mois-

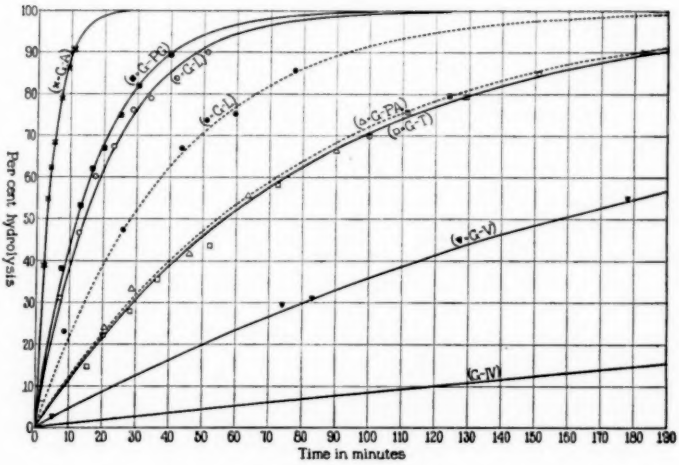

FIG. 1. Hydrolysis of ketopiperazines by alkali. Curves give calculated values, and points are experimental. Broken line curves represent hydrolysis with pyridine present. G-A indicates glycyl-dextro-alanine anhydride; G-PG, glycyl-levo-phenylglycine anhydride; G-L, glycyl-levo-leucine anhydride; G-PA, glycyl-dextro-phenylalanine anhydride; G-T, glycyl-levo-tyrosine anhydride; G-V, glycyl-*d,l*-valine anhydride; G-IV, glycyl-dextro-isovaline anhydride.

ture) of anhydride was weighed in a 25.0 cc. volumetric flask, 8.0 cc. of pyridine and 12.5 cc. of 1.0 N NaOH (5 mols per mol of anhydride) were added. The volume was made up to 25.0 cc. with water. The extent of hydrolysis was determined by titration in the neutralizing solutions used in connection with this method. The free pyridine in the mixture did not affect the titration.

All data are summarized in Tables II and III.

Constants expressing the rates of hydrolysis were calculated by the equation

$$kt = \log_{10} \frac{a}{a - x}$$

The calculations were made on the assumption that no hydrolysis of the formed peptides to the amino acids occurred; indeed, this secondary hydrolysis is practically nil under these conditions. By means of the average constants, hydrolysis curves were constructed for each ketopiperazine (Fig. 1). The average constant $k' \cdot 10^3$ established for glycyl-dextro-phenylalanine anhydride can obviously not be compared with the average constants $k \cdot 10^3$ of the other ketopiperazines. That the hydrolysis of ketopiperazines by alkali proceeds much slower when pyridine is present in the mixture is shown by the parallel experiment performed under identical conditions on glycyl-levo-leucine anhydride. For this compound $k' \cdot 10^3$, thus found, is $10._7$, while $k \cdot 10^3$ found with purely aqueous alkali is $20._8$. It should be noted that the constants given represent the resultants of the rates of formation of the two possible isomeric dipeptides and that in the case of glycyl-dextro-alanine anhydride, the rate of hydrolysis is so rapid that a slight error in the time interval introduces a considerable error in the constant. The agreement in the data is as good as can be expected under the conditions which have been used. Furthermore, the change in the pH of the hydrolysis solutions, due to the buffering action of the peptide formed, has an influence on the reaction constants. Also, in the case of glycyl-levo-tyrosine anhydride the phenolic hydroxyl consumes up to 1 equivalent of sodium hydroxide. This must be taken into consideration in any comparison of hydrolysis constants.

THE OXIDATION-REDUCTION POTENTIALS OF HEMIN AND RELATED SUBSTANCES.

I. THE POTENTIALS OF VARIOUS HEMINS AND HEMATINS IN THE ABSENCE AND PRESENCE OF PYRIDINE.

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(Received for publication, February 10, 1930.)

As a continuation of our electrochemical study of hemin and related substances (1, 2) we have measured the oxidation-reduction potentials in borax-tartrate buffer solutions by the titration method. The ferric compound was titrated with titanous chloride in the manner previously described; in a few cases the titration of the ferrous compound with ferricyanide was also completed.

At constant hydrogen ion activity at 25° the potential of an oxidation-reduction system involving 1 equivalent change is given by Equation 1.

$$E = E_0' - 0.06 \log \frac{[\text{Red}]}{[\text{Ox}]} \tag{1}$$

The value of E_0' (the normal potential at the pH employed) is the value of the potential (referred to the normal hydrogen electrode) at the mid-point of the titration curve. This value, in the case of the hemin and hematin systems, we have found to be fairly reproducible and we feel confident that we have evaluated it within 10 millivolts for the particular systems we have studied. The data summarized in Table I were obtained at 22° ±2° with a buffer solution composed of equal amounts of 0.1 M borax and 0.2 M sodium tartrate; its pH value was 9.15. It is evident that E_0' for both the hemin and hematin systems is identical within the limits of experimental error. The hemin employed was crys-

talline α-hemin prepared by the usual acetic acid method and recrystallized by means of the chloroform-pyridine treatment (3). We shall refer to this material throughout this paper as hemin. Experiments were also performed with β-hemin ((4), (5) p. 203–205) prepared by the acetone and by the alcohol procedure. Various varieties of hematin were also employed: alkali hematin (6), Küster's hematin ((5) p. 210), Mörner's hemin ((5) p. 203–205), and Verdaung's hematin (7). All these materials gave essentially the same value for E_0' in the borax-tartrate buffer.

TABLE I.

Determination of Normal Potential of Hemin-Reduced Hemin and Hematin-Reduced Hematin Systems.

Concentration = 2×10^{-4} molar; temperature = 22° ± 2°.

pH	Method.	No. of determinations.	Highest value E_0'.	Lowest value E_0'.	Average values E_0'.	Mean of E_0'.
	A. Hemin system; hemin dissolved at 22°.					
			volts	*volts*	*volts*	*volts*
9.15	$TiCl_3$	3	−0.224	−0.244	−0.235	−0.230
	$K_3Fe(CN)_6$	2	−0.224	−0.224	−0.224	
	B. Hemin dissolved at 70° in borate buffer.					
9.15	$TiCl_3$	4	−0.223	−0.241	−0.237	−0.230
	$K_3Fe(CN)_6$	1			−0.224	
	C. Hematin systems; hematin dissolved in borate buffer at 23°.					
9.15	$TiCl_3$	2	−0.241	−0.244	−0.242	−0.242

A few experiments with a buffer solution of pH 10.26 showed that the potential becomes more negative with increasing pH values. Further experimentation will be necessary to formulate and explain the change of E_0' with pH.

An inspection of the typical titration curves published in our last paper (2) shows that the form is far from regular. In spite of great pains and much work we have been unable to obtain consistent and reproducible slopes of the titration curves either in the absence or presence of pyridine. The irregularities may be best described by noting that the difference in potential between

the 20 per cent reduction point and the mid-point varied from 15 to 120 millivolts, a value of 60 millivolts being most representative. The difference in potential between the mid-point and the point of 80 per cent reduction showed less fluctuation; the extremes were 40 and 70 millivolts, while many results were very near 50 millivolts. The theoretical is 36 millivolts in each case. Thus, the *shape* of the E.M.F. curves is less consistent than the value of E_0', and the curves on the whole are steeper than would be predicted from Equation 1.

The two experimental difficulties which are reflected in these unsatisfactory results are: (1) extreme sensitivity to oxygen and (2) unsatisfactory electrode equilibria. Probably both difficulties are accentuated by the low molecular concentration which is often necessitated by the insolubility of the compounds. The sensitivity to oxygen of reduced hemin or hematin solutions is very much greater than that of any other compounds which have been studied in this laboratory. Alkaline solutions of anthrahydroquinone derivatives are very sensitive to oxygen. Nevertheless, nitrogen which has been purified by passing through such a solution (Fieser's method) still contains sufficient oxygen to cause a "drift" in potential of partially reduced hemin. Even passing the nitrogen over heated copper was often not entirely satisfactory.

Two bright platinum electrodes were employed in all the experiments. They were cleaned, scoured, and reheated from time to time. In most titrations the electrodes agreed within a few millivolts except at the beginning and the end of the titration, but this agreement was more difficult to obtain than in the measurements of most reversible systems. It is strange that, in spite of the difficulties of obtaining really accurate measurements at a concentration of 10^{-4} molar, fairly definite and reproducible measurements can still be obtained at a concentration of only 10^{-6} molar. At this same low concentration, many reversible systems no longer impart a definite potential to an inert electrode.

The effect of changing concentration on the hemin or hematin systems appears to be enormous, the potential becoming more positive on dilution. Thus, at a concentration of 1.9×10^{-4} molar the value of E_0' for both hemin and hematin is about -0.230, while at a concentration of 3.8×10^{-5} the value for hemin is -0.164, and at a concentration of 1.9×10^{-5} is -0.104

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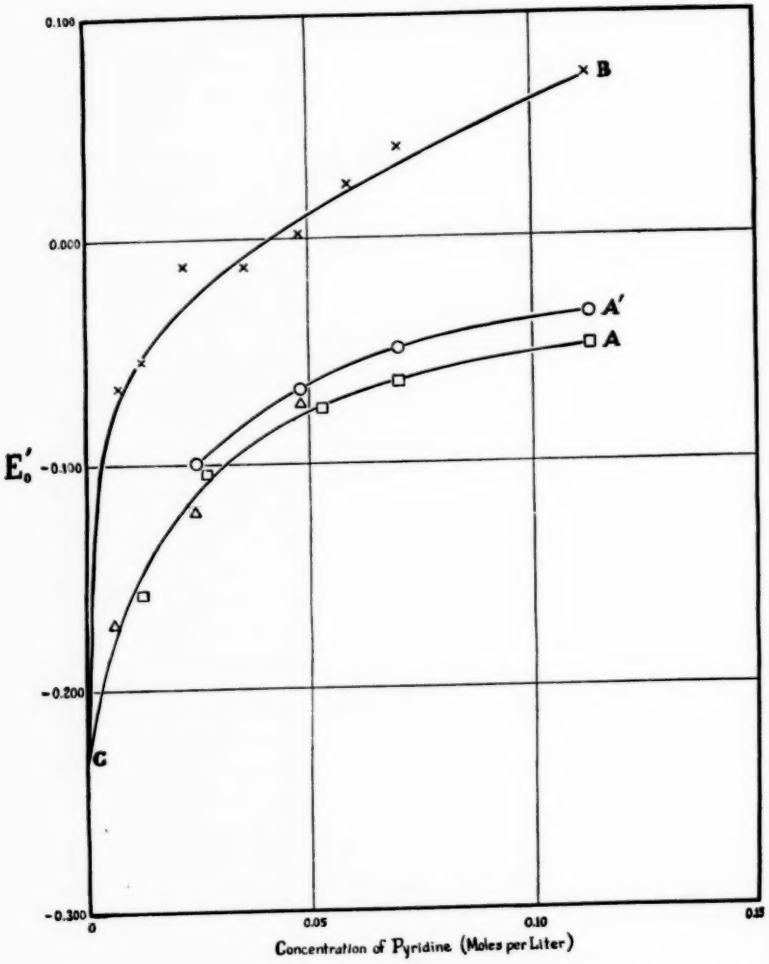

FIG. 1. The oxidation-reduction potential of the hemin and hematin systems in the presence of pyridine. Curve AC gives the values of E_0' for hemin hydrolyzed in 0.1 M borax at 70° for 30 minutes = □ and hematin = Δ, concentration 1.9×10^{-4} molar. Curve A′ shows the effect of a 5-fold dilution. Curve BC represents data obtained in the experiment noted in Table II. In each case a smooth curve has been drawn through the experimental points.

for hematin. These large changes, if they are real and not due to some undiscovered experimental error, must be due to the fact that either the ferric or ferrous compound (or both) must be considerably associated. This association may well be due to the tendency of the iron atom to draw to itself other basic nitrogenous substances. Since dilution would favor the dissociation of the complex, and since dilution raises the potential, it seems clear that the ferric compound must be more associated than the ferrous compound under comparable conditions.

Effect of the Addition of Pyridine.

The addition of small amounts of pyridine to a reduced hemin or hematin solution makes the potential more positive. The change continues with increasing pyridine concentration. This is shown in Column 4 of Table II and in Fig. 1. A comparison of Curves BC and AC shows clearly that hemin is changed by heating with borax at 70° for 30 minutes. This change probably involves the replacement of the chlorine atom of hemin by a hydroxyl group and may involve a rearrangement of the linkages in the porphyrin molecule. If the hemin is heated in the borax solution for shorter periods of time and then titrated with titanous chloride in the presence of pyridine, the normal potential lies between that of the hemin-pyridine and hematin-pyridine systems. These results show that in employing alkaline solutions of hemin one may be dealing with solutions of hemin or a hydrolysis product depending on the alkalinity, the temperature, and the time during which the solution has stood. Hemin heated in borax is practically identical to hematin in its behavior towards pyridine as shown by the points plotted in Fig. 1. As will be shown below, they are not identical substances, however.

The most significant results given are those obtained with a concentration of 1.9×10^{-5} mols per liter. However, in the case of the hemin-pyridine system, there was some precipitation of pyridine-hemochromogen during the titration. There was less precipitate at a concentration of 0.38×10^{-5} molar, but the difficulty of obtaining satisfactory end-points at this concentration makes the results less reliable. It will be noted that in both the hemin-pyridine and hematin-pyridine systems the effect of dilution is to make the potentials somewhat more positive;

the effect is relatively slight, however, compared with the enormous effect of dilution in the absence of pyridine.

There is considerable spectroscopic and other evidence which shows that pyridine combines with both hemin and reduced hemin. The combination with the latter substance is known as pyridine hemochromogen. The equilibrium in this reaction has been studied by Hill (8) and more recently by Anson and Mirsky (9) who have questioned the validity of Hill's simple formulation of the reaction. Qualitatively the effect of pyridine on the potential of the hemin or hematin system is just what would have been predicted. The combination between the ferrous compound and

TABLE II.

*Effect of Addition of Pyridine on Normal Potential of the Hemin System.**

Hemin concentration 0.38×10^{-4} molar; temperature = 22° ± 2°.

1.24 M pyridine in 100 cc.	Concentration of pyridine.	Normal potential E_0'.
cc.	*M*	*volts*
3.00	0.0361	+0.001
5.00	0.0590	+0.011
10.00	0.113	+0.056

The hemin was dissolved at room temperature in 50 cc. of 0.1 M borax to which had been added the amount of 1.24 M pyridine solution shown in the first column; 50 cc. of 0.2 M sodium tartrate were then added and the titration with titanous chloride performed in the usual manner. The value of E_0' was the value of E at the mid-point.

* The data for a similar experiment on hemin of 1.9×10^{-4} molar concentration are indicated by Curve BC, Fig. 1.

pyridine being more complete, the potential is raised by the addition of increasing amounts of pyridine. A quantitative treatment is much more difficult, particularly if we attempt to explain by means of association the effect of dilution on the potentials in the absence of pyridine. However, if we neglect association, we may derive an equation for the relationship of E_0' to the concentration of pyridine as follows:

Let $[Py]$ = concentration of pyridine, $[\text{Ferric}]_T$ and $[\text{Ferrous}]_T$ the *total* concentrations of oxidized and reduced pigment respectively and assume activities equal to concentrations, then we may

write for the two equilibria the mass law constants K_r and K_0 respectively:

$$\frac{[\text{Ferric complex}]}{[\text{Ferric}]\,[Py]^n} = K_0 \tag{2}$$

$$\frac{[\text{Ferrous complex}]}{[\text{Ferrous}]\,[Py]^m} = K_r \tag{3}$$

It thus follows that $[\text{Ferric}]_T = [\text{Ferric}] + K_0\,[\text{Ferric}]\,[Py]^n$ and $[\text{Ferrous}]_T = [\text{Ferrous}] + K_r\,[\text{Ferrous}]\,[Py]^m$.

Substituting the values of [Ferric] and [Ferrous] for the [Ox] and [Red] terms in Equation 1, we have Equation 4:

$$E = E_0' - 0.06 \log \frac{[\text{Ferrous}]_T}{[\text{Ferric}]_T} - 0.06 \log \frac{1 + K_0\,[Py]^n}{1 + K_r\,[Py]^m} \tag{4}$$

It is evident that when $[Py] = 0$ Equation 4 reduces to Equation 1. If we assume, further, that K_r is much larger than K_0 (an assumption for which there is a good deal of evidence), then for the lower values of $[Py]$ we may write:

$$E = E_0' - 0.06 \log \frac{[\text{Ferrous}]_T}{[\text{Ferric}]_T} + 0.06 \log K_r\,[Py]^m \tag{5}$$

The last term may be taken as corresponding to the increase of potential with increasing pyridine concentration at least for the first portion of the curve shown in Fig. 1. If we take a value for K_r of 10^5 and $m = 2$, a very fair agreement with the experimental data is obtained, as illustrated below in the case of the hydrolyzed hemin or hematin data.

Concentration of pyridine. *Mol per l.*	Difference in E_0' from E_0' when $[Py] = 0$ Found.	Calculated $K_r = 10^5$
0.01	0.060	0.060
0.02	0.104	0.096
0.03	0.128	0.118
0.05	0.150	0.144
0.10	0.180	0.180

Very similar results may be obtained by an application of Equation 5 to the results obtained with hemin dissolved in borax (Table II). Taking $m = 2$, the values of $\log K_r$ found from the

experimental data range from 6.60 to 6.83 with a mean of 6.71. In these calculations no correction has been made for the effect of the added pyridine on the pH value of the buffer solution.

While the fact that by the use of one constant and several reasonable assumptions it is possible to derive a theoretical equation which fits the experimental data is gratifying, it cannot be said to constitute a proof of the correctness of our assumptions. Further work which is now in progress on the effect of dilution on all the systems must be considered before a final answer to the problem may be given.[1]

In conclusion we may mention a few preliminary results of our study of the action of potassium cyanide which is still in progress. It is well known that the cyanide ion combines with hemin and reduced hemin and the resulting products may be characterized spectroscopically. The affinity between cyanide and the iron compounds is much greater than that between pyridine and the same compounds (see Anson and Mirsky (9) and Hill (8, 10)). In keeping with these facts we have found that a concentration of only 0.04 molar of cyanide is sufficient to produce the maximum effect on the potential systems in borax-tartrate solutions. The lower limits of the reaction have not yet been investigated. If hemin is dissolved in the borax buffer in the presence of this amount of cyanide and at room temperature, the resulting value of E_0' is about -0.160 (an increase of about 70 millivolt from the value in the absence of cyanide). β-Hemin (from acetone) gave the same figure, while the various varieties of hematin and β-hemin (alcohol) gave a slightly lower value (-0.190). In all these experiments the concentration of pigment was 1.9×10^{-4} molar; at one-tenth this concentration about the same values were obtained. Strangely enough, if hemin or hematin is heated with the borax solution for only a few minutes at 70°, the sub-

[1] Since this paper was prepared for publication, the recent results of Hill (10) have appeared. In this paper Hill replies to the criticisms of Anson and Mirsky and gives additional data concerning the pyridine-hemochromogen equilibrium. His value for $K = \frac{1}{K_r}$ is 8.9×10^{-6}. This is probably to be compared with our value for the hematin system of 10^{-5} for $\frac{1}{K_r}$. The agreement is all that could be expected.

sequent addition of cyanide has no effect on the value of E_0' although a complex is formed as shown by the color. If cyanide is present during the heating, the usual complex is formed (judged by the effect on the potential). Attention was drawn earlier in this paper to the fact that α-hemin hydrolyzed by heating with borax behaved similarly to hematin in regard to the action of pyridine. The experiments with cyanide, however, show that in reality the two compounds are different.

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STUDIES IN THE CHEMISTRY OF VITAMIN A.

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INTRODUCTION.

In 1925, Bills (1) reported observations on the action of various reagents on the antirachitic vitamin. It occurred to us that an extension of this work to vitamin A might be of value in elucidating its chemical nature.

EXPERIMENTAL.

The animals used in the research were standard albino rats and a strain of hooded rats which had been inbred for a number of years. The stock members of the colony were maintained on Steenbock's ration (2) except that bone ash was substituted for calcium carbonate in accordance with later recommendations of the author. Fresh, whole milk was given *ad libitum*. Litters were reduced to six animals.

In testing for vitamin A the growth method of Drummond and Coward (3) and the curative method of Steenbock and Coward (4) were employed. The basal vitamin A-free ration consisted of fat-free heated casein, 18 per cent; dried yeast, 6 to 8 per cent; Steenbock's Salts 40 (5), 4 per cent; dextrinized starch (6), 70 to 72 per cent; distilled water, *ad libitum*. The antirachitic factor was supplied by irradiation of the casein before a quartz mercury vapor lamp of the Hashimoto and Wu type (7). Later in the work the dextrin also was irradiated.

The experimental diet consisted of the basal vitamin A-free ration in which the substance to be tested had been incorporated by adding it at the expense of an equal percentage of dextrinized starch. The progress or cure of the xerophthalmia was deter-

mined by frequent examination of the animals. The effect on growth was observed by weekly weighings. The dry food was presented to the animals in metal cups with suitable traps to avoid spilling, thus permitting the exact determination, by weighing, of the food consumed.

A. Sulfur Dioxide.

Of the reagents examined by Bills (1), sulfur dioxide was found to be without action on the antirachitic vitamin. Early in our investigations, however, we found the effect of this substance on vitamin A in cod liver oil to be of such a magnitude as to render desirable an examination of vitamin A from other sources. In doing so we were guided by two principal considerations. In the first of these we had in mind the query as to whether vitamin A activity is the property of a single chemical individual or that of a specific atomic grouping possessed by several different molecules. The latter view was advanced recently by von Euler, von Euler, and Hellström (8). The second consideration was a question of economic importance. On the assumption that vitamin A of the green plant would be as susceptible to destruction by sulfur dioxide as vitamin A of animal origin, it occurred to us that one important aspect of the damage to vegetation by smelter smoke would be the destruction of vitamin A. With these considerations before us we examined the effect of sulfur dioxide on the vitamin A content of cod liver oil, butter, alfalfa, and spinach.

Commercial sulfur dioxide, purified by washing with water and concentrated sulfuric acid, was employed. Cod liver oil was treated by bubbling with the purified gas (125 to 150 bubbles per minute through a 4 mm. tube) at a temperature of 20–100° for periods of 15 minutes to 2 hours. The excess of sulfur dioxide was removed by exposure of the treated oil *in vacuo* for several hours.

Butter was first melted at 60° and filtered through glass wool to remove casein. The water was drawn off through a separatory funnel. The product was divided into two portions one of which was reserved unsulfured for control experiments. The other was treated by bubbling with sulfur dioxide at 60° for 1 to 24 hours. Excess sulfur dioxide was removed *in vacuo* at 60°.

In preliminary experiments alfalfa and spinach were treated in

TABLE I.

Effect of Sulfur Dioxide on Vitamin A Activity of Oils.

TABLE I.

Effect of Sulfur Dioxide on Vitamin A Activity of Oils.

Oil.	Treatment with SO_2.	Oil ingested per rat.		Food ingested.	Xerophthalmia.	Growth.	Duration of experiment.
		mg. per day	*per cent of ration*	*gm. per day*			*wks.*
Cod liver oil.	None.	19	0.25	7.5	Cured in 1 to 4 wks.	Good.	16
" " "	15 min. at 20°.	23	0.5	4.5	Blind.	Very poor. One died.	9
" " "	1 hr. " 60°.	15	0.25	6.0	"	" " Two "	10
" " "	2 hrs. " 100°.	230	5.0	4.6	"	" " Two "	8
Butter.	None.	160	2.0	8.0	Cured in 2 to 3 wks.	Very good.	10
"	22 hrs. at 60°	405	5.0	8.1	" " 1 " 2 " *	Good.	11
"	22 " " 60°.	108	2.0	5.4	Blind.	Very poor. Two died.	10
"	2 " " 60°.	320	5.0	6.4	Cured in 1 wk.	Good.	8
"	2 " " 60°.	86	2.0	4.3	Blind.	Very poor. Two died.	10
"	None.	79	1.0	7.9	Slight.	Poor.	12
Alfalfa.	None.	435	5.0	8.7	Cured in 1 wk.	Very good.	10
"	1 hr. at 60°	305	5.0	6.1	" " 1 "	Good.	10
"	None.	122	2.0	6.1	" " 2 to 4 wks.	Fair to good.	11
"	1 hr. at 60°.	98	2.0	4.9	" " 1 wk.	Fair.	11
"	None.	42	1.0	4.2	Nearly blind.	Very poor. All died.	10
"	1 hr. at 60°	32	1.0	3.2	" "	" " " "	10
Cod liver oil diluted with inactive cottonseed oil.	None.	42	0.5	8.5	Cured in 1 wk.	Very good.	9
	1 hr. at 100°.	25	0.5	4.9	Nearly blind.	Very poor.	9
	2 hrs. " 100°.	51	1.0	5.1	" "	" "	9

* Xerophthalmia reappeared in two animals.

the dry and green condition. This was done by placing the dried, ground, or fresh leaves in thin layers on trays contained in a chamber in which sulfur dioxide was generated by burning sulfur. After ½ hour the product was removed and left in the open air overnight.

In later experiments, where a rigid comparison between the stabilities of vitamin A from different sources was necessary, an extract of alfalfa was employed which could be treated in precisely the same way as cod liver oil. This was accomplished by the use of a concentrated alcoholic extract of alfalfa "leaves and blossoms."

119 animals were used in the experiments on cod liver oil, 54 on butter, and 103 on alfalfa and spinach. The results are presented in Table I in which each line presents the results gained on a group of four animals. The experiments on dry alfalfa and spinach have been omitted because the conditions of treatment with sulfur dioxide were not strictly comparable to those pertaining to cod liver oil and butter. It is sufficient to point out that no destruction of vitamin A in the green or dry alfalfa and spinach was observed.

B. Sodium Bisulfite.

The inactivation of vitamin A in cod liver oil by treatment with sulfur dioxide suggested that sodium bisulfite might likewise be destructive. This would be especially probable if vitamin A possesses the aldehyde properties attributed to it by some investigators (9).

60 gm. of purified sodium bisulfite were mixed with 100 cc. of cod liver oil, placed in a stoppered bottle, and maintained at 100° for 4 to 27 hours. At the end of the experimental time the oil, now reddish brown in color, was separated from the excess sodium bisulfite by filtration. The results indicated that prolonged treatment (24 hours) with sodium bisulfite destroys vitamin A. 4 hours treatment produced no apparent loss.

C. Hydrogen Sulfide.

Hydrogen sulfide, generated by the action of sulfuric acid upon ferrous sulfide, was washed with water and preheated in a copper coil to 100°. Cod liver oil was treated with the gas by bubbling at 100° for 6 hours. At the end of this time the excess of hydro-

gen sulfide was removed by warming the oil *in vacuo* at 70° for 2 hours. When fed at a level of 0.5 per cent significant destruction of vitamin A could be observed.

D. Hydrogen Peroxide.

The fact that vitamin A may be destroyed by atmospheric oxygen suggested the use of hydrogen peroxide in this series of experiments.

50 cc. of cod liver oil were shaken with 150 cc. of high quality hydrogen peroxide for 5 minutes daily over a period of 18 days. An emulsion formed which persisted for several hours. The mixture was kept in the dark in a stoppered bottle. After 18 days the oil was separated by centrifuging. Partial destruction of vitamin A was accomplished.

E. Phosphorus Pentachloride.

As a means of investigating the hypothesis (9) that vitamin A in cod liver oil possesses aldehydic properties a number of reagents, known to react with aldehydes, were examined. Among these phosphorus pentachloride was tried. 100 cc. of cod liver oil were mixed with 50 gm. of phosphorus pentachloride. The mixture was allowed to stand at room temperature for 22 hours. The oil was then decanted from the solid pentachloride and washed several times with distilled water until the hydrochloric acid produced by hydrolysis was removed. The product was deep purple in color and possessed a sharp, penetrating odor. Complete destruction of vitamin A was observed.

F. Chlorine.

Cod liver oil was treated with chlorine at 100° by bubbling with the preheated gas. One sample was treated for 15 minutes and a second for ½ hour. The two samples were then maintained for 4 hours *in vacuo* at 60–70° for removal of excess chlorine. Under these conditions vitamin A was destroyed.

G. Acetyl Chloride.

100 cc. of cod liver oil were mixed with 100 cc. of acetyl chloride. A reddish brown solution formed, which was kept at room temperature for 22 hours. At the end of this time the solution was

poured into distilled water, thus hydrolyzing the excess acetyl chloride and yielding copious fumes of hydrogen chloride. The oil was removed with a separatory funnel and washed freely with portions of distilled water. The vitamin A activity of the oil was found to be destroyed.

H. Ethylene.

This gas was examined because of its established potency in inducing physiological changes in plants (ripening of fruits) and animals (anesthesia).

100 cc. of cod liver oil were treated with ethylene for 6 hours at 100°. The gas was drawn from a commercial cylinder, preheated, and passed through the oil. No destruction of vitamin A could be observed.

I. Nitrous Fumes.

The fact that nitrous fumes were found by Bills (1) to be destructive to the antirachitic vitamin suggested an examination of their effect on vitamin A. The fumes were generated by the action of 20 per cent acetic acid on sodium nitrite at room temperature. The gas was heated to 100° and passed through cod liver oil heated to the same temperature. Portions of the oil were treated for ½ and 6 hours respectively. The excess gas was removed *in vacuo*. Vitamin A was found to be destroyed.

J. Benedict's Alkaline Copper Reagent.

On the assumption that vitamin A of cod liver oil exhibits aldehydic properties as contended by Takahashi (9), it seemed of interest to examine the action of Benedict's reagent which is known to effect the oxidation of many of the higher aliphatic aldehydes.

100 cc. of cod liver oil and 150 cc. of Benedict's alkaline copper solution, as used for the qualitative determination of reducing sugar, were shaken together. A similar portion, designed to serve as a control, was treated with Benedict's solution which had previously been rendered slightly acid by the addition of sulfuric acid. Both portions were kept in stoppered bottles for 24 hours at 100°. At the end of this time the oils were well washed and separated from excess water by centrifuging. Cuprous oxide had separated out from the alkaline sample while no reduction of the copper

could be observed in the control acidified portion. The vitamin A activity of the former was found to have been destroyed. That of the latter was unchanged.

K. Formaldehyde.

Formaldehyde was generated by heating high grade formalin. Water vapor was removed by condensation. The gas was heated to 100° and passed through cod liver oil for 1 hour at 100°. When fed at a level of 0.5 per cent destruction of the vitamin was apparent. It is, however, to be observed that the amount ingested (23 mg. per day) is little more than enough to provide the requirement of vitamin A assuming no destruction to have taken place. When fed at a higher level, such that 112 mg. were ingested per day, little destruction of the vitamin could be observed.

L. Ammonia.

150 cc. portions of cod liver oil were treated with ammonia gas at 100° for 6 and 25 hours respectively. The excess ammonia was removed *in vacuo* at 70°. In the 25 hour sample a precipitate appeared after standing a few days at room temperature. This was filtered off. The residue thus removed and the filtrate were tested separately for vitamin A, the former after being washed and suspended in inactive cottonseed oil. No destruction of vitamin A could be observed.

DISCUSSION.

In cod liver oil, treatment with sulfur dioxide for as short a time as 15 minutes at room temperature was sufficient to cause a marked loss of the growth-promoting, antixerophthalmic activity. At 100° the destruction was complete as indicated by feeding the treated oil liberally (5.0 per cent of the whole ration). Under fairly similar conditions (1 hour at 60°) the destruction of vitamin A in alfalfa extracts was inappreciable. Butter occupied a somewhat intermediate position. Though treated for 2 hours at 60° and fed at a level of 5 per cent, the extract continued to show marked vitamin A activity. Even after 22 hours of treatment much of the activity remained. When fed at the lower level of 2 per cent, partial destruction of the vitamin became apparent. Nevertheless, it must be noted that of the material treated for 2 hours and

fed at a level of 2 per cent only 86 mg. of the oil were ingested, while control experiments showed that 79 mg. of untreated oil were hardly sufficient to cure the xerophthalmia. The effects on growth are probably due in part to the difference in food consumption (4. 3 gm. *versus* 7.9 gm.).

Apparently the vitamin A activity of cod liver oil is to be regarded as the property of a substance or substances not contained in significant quantities in butter and extracts of alfalfa, or else the vitamin A of plant origin is associated with a protective substance not found in cod liver oil. The latter possibility is supported by the interesting observations of King, Rosenheim, and Webster (10) on ergosterol, that of Steudel (11) on vitamin A in brain tissue, and that of Mattill (12), Estill and McCollum (13), and Huston, Lightbody, and Ball (14), on antioxidants as protectors of vitamin A. We are, however, inclined to give greater weight to the hypothesis first mentioned, which is in effect that vitamin A in cod liver oil is qualitatively different from vitamin A in alfalfa. In view of the recent important observations of von Euler, von Euler, and Karrer (15) on the vitamin A activity of carotene and dihydro-α-crocetin, and the inactivity of several other carotenoids, much may be said in favor of the hypothesis that vitamin A activity is the property of a definite atomic grouping, possessed by different chemical individuals. The careful work of von Euler, von Euler, and Hellström (8), Moore (16), and Collison, Hume, Smedley-MacLean, and Smith (17), would almost compel one to attribute vitamin A activity to certain of the plant carotenoids were it not for the recent negative results of Drummond and associates (18).

On the other hand von Euler, von Euler, and Hellström (8) have been careful to point out that in their judgment the vitamin A activity of cod liver oil may not be due to a carotenoid. There are also the spectroscopic findings of Moore (16), von Euler, von Euler, and Hellström (8), and Dulière, Morton, and Drummond (19) on the blue antimony chloride-carotene product which agree in locating the principal absorption band at 590μμ. There is no evidence of absorption at 610μμ which is the zone of absorption characteristic of the antimony chloride-cod liver oil product. Finally, mention should be made of the observations of Sherman, Quinn, Day, and Miller (20) in which vitamin A of spinach extract

was found to be somewhat more thermostable in an atmosphere of nitrogen than that of a similar extract of butter.

An examination of the effects produced by the other agents on the vitamin A activity of cod liver oil permits at least one conclusion as to the nature of the active principle. The observed loss of vitamin A activity on treatment of cod liver oil with sulfur dioxide and Benedict's reagent, suggests to us that the active principle possesses aldehydic properties. This is supported by the results of similar nature which followed the use of phosphorus pentachloride and prolonged treatment with sodium bisulfite. The results with hydrogen peroxide and chlorine are also compatible with this point of view. We are inclined to attribute the destructive action of chlorine to its activity as an oxidizing agent although no conclusive evidence can be offered in support of this opinion. The failure of ammonia to inactivate the vitamin is nevertheless difficult to reconcile with the aldehyde hypothesis, for under these conditions the formation of an aldehyde-ammonia compound might reasonably be expected.

It is of course apparent that loss of vitamin A activity may not in all instances be attributed to inactivation of a single essential group in the molecule. No matter how much evidence may be marshalled in the support of the theory that the active principle contains an aldehyde group, it is always possible that the destructive action of various reagents may in some instances be due to their effects upon a second or third group. Thus it seems more acceptable to attribute the loss of vitamin A activity which attended the use of acetyl chloride, to acetylation of hydroxy groups than to any action on the hypothetical aldehyde group.

The failure of hydrogen sulfide and, to a limited extent, of formaldehyde to inactivate vitamin A is in harmony both with the known stability of this principle towards reducing agents and the fact that aldehydes are not readily reduced by these substances under the conditions of treatment adhered to in this investigation.

The destructive action of nitrous fumes may be due to their oxidizing power, although when cognizance is taken of the many types of reactions in which nitrous fumes participate, much caution is needed in deducing conclusions on structure.

Acting on the hypothesis that vitamin A of cod liver oil is an aldehyde, we conceived of the destructive action of sulfur dioxide

and sodium bisulfite as being due to the formation of a sulfite addition compound. Inasmuch as aldehydes in the form of such compounds can commonly be regenerated by treatment with carbon dioxide, experiments were conducted to test this possibility.

100 cc. portions of cod liver oil, inactivated by treatment with sulfur dioxide for 1 hour at 65°, were bubbled with carbon dioxide for 1, 9, and 24 hours respectively at 100°. The product was incorporated in the basal ration at a level of 1 per cent and fed to eight rats. An average of 43 mg. was ingested daily by each animal without curing the xerophthalmia or exercising any beneficial effect on growth. Regeneration of the factor was not accomplished.

We assumed that the destructive action of sulfur dioxide might possibly be through oxidation although this appealed to us as somewhat improbable. Nevertheless, we resorted next to the use of a reducing agent in attempts to regenerate the vitamin. Hydrogen in the presence of a nickel catalyst was used. We examined its action on oil which had been inactivated by treatment with sulfur dioxide and, for comparative purposes, on a sample inactivated by atmospheric oxygen. In the latter case destruction of vitamin A was accomplished by aeration for 20 hours at 100°. Treatment with hydrogen was carried out at 100° for 2, 6, and 24 hours. The products were fed to groups of rats at suitable levels but no restoration of the vitamin A activity was observed.

SUMMARY.

1. Cod liver oil, butter, and a concentrated alcohol extract of alfalfa were treated with sulfur dioxide. The effects on the growth-promoting and antixerophthalmic activities of the materials were determined.

2. The active principle of cod liver oil was rapidly and completely destroyed by sulfur dioxide. That of alfalfa suffered no appreciable destruction. The vitamin A activity of butter was lessened.

3. Alfalfa and spinach, when sulfured in the dry and green conditions, experienced no loss of vitamin A activity.

4. Phosphorus pentachloride, chlorine, acetyl chloride, nitrous fumes, and Benedict's alkaline copper reagent destroyed the active principle in cod liver oil. Prolonged treatment with sodium

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bisulfite had the same effect. Hydrogen sulfide, ethylene, ammonia, and Benedict's reagent after neutralization exhibited no destructive action. Formaldehyde had little effect. Hydrogen peroxide brought about a partial loss.

5. Attempts to regenerate the vitamin A potency of oils that had been inactivated by treatment with sulfur dioxide and oxygen were unsuccessful.

6. It is concluded that vitamin A activity is the property of a specific atomic grouping rather than of a specific molecule. The active principle in cod liver oil is probably not identical with that in alfalfa. An alternative explanation is that alfalfa contains substances, absent from cod liver oil, that exercise a protective function against the destructive action of sulfur dioxide. We regard the latter explanation as less acceptable than the former.

7. The active principle of cod liver oil possesses aldehyde properties.

We are indebted to Professor R. E. Swain for many helpful suggestions received in the course of this investigation and to Mead Johnson and Company for gifts of cod liver oil.

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CAROTENE.

II. THE VOLATILE FATTY ACIDS OBTAINED BY THE OXIDATION OF CAROTENE AND XANTHOPHYLL.

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(Received for publication, February 11, 1930.)

During the preliminary stages of our investigations on the structure of carotene, acetic acid was identified as a product of oxidation of this hydrocarbon (1). It soon became evident that the quantitative determination of the volatile acids formed by the oxidation of carotene would yield valuable information concerning its structure. However, satisfactory quantitative results were not obtained until a procedure was developed whereby this oxidation could be carried out in a homogeneous system. This, as has been recently described (2), was accomplished by the use of pyridine in which both carotene and potassium permanganate are sufficiently soluble.

The quantitative determination of the number of mols of acetic acid formed on oxidation, in conjunction with further observations which have been made on other members of this group, contributes directly to the problem of the structure of carotene.

Experimental Procedure.

A sample of carotene was weighed into a 150 cc. glass-stoppered Erlenmeyer flask and dissolved in 3 cc. of purified pyridine.[1] To this was added 50 cc. of an approximately 0.1 N solution of potassium permanganate in this same solvent. The flask was stoppered and placed in a constant temperature bath at 37.5°

[1] Merck's medicinal pyridine was purified for this purpose by distilling from potassium permanganate, then from lime, and finally redistilling, the fraction between 112° and 115° being used in these experiments.

for 4 hours, a period known by experience to be sufficiently long to complete the oxidation of the carotene. At the end of this period, the reaction mixture was transferred quantitatively to a 300 cc. Erlenmeyer flask and the reaction vessel rinsed with an equal volume of water. Granulated aluminum, 3 to 5 gm., and mercury, 10 to 15 gm., were added to the solution in order to reduce the excess permanganate. The reduction was complete in about an hour. (Later it was found that the reduction could be more quickly accomplished by mixing the aluminum and mercury previous to the addition of the reaction mixture.) When the last trace of pink color had disappeared the manganese dioxide was coagulated by heating to incipient boiling and the solution filtered into a 300 cc. Claissen flask. The solvent was then removed by distillation under diminished pressure and the last traces of pyridine swept away by distilling an additional 50 cc. portion of water. In the reduction of the potassium permanganate, potassium hydroxide is, of course, formed so that the organic acids are here present as the potassium salts.

The residue from the oxidation, after removal of all the pyridine, was dissolved in water and transferred quantitatively, with as little water as possible, to an apparatus especially designed for the estimation of small quantities of volatile acids. The solution was concentrated under diminished pressure to about 2 cc. The volatile acids were liberated by the addition of 0.5 to 1.0 cc. of phosphoric acid (sp. gr. 1.71) and distilled under reduced pressure. They were condensed by a silver condenser and collected in a quartz receiver containing an accurately measured quantity of 0.01 N sodium hydroxide. To the residue in the distilling flask was added approximately 1 cc. of water and this was distilled. Four or five such additions of water and subsequent distillations transferred the residual portions of the volatile acids to the receiver. The condenser tube was rinsed and the excess alkali in the quartz receiver was titrated with 0.01 N acid. From this titration the volatile acids were estimated.[2]

[2] In order to remove the carbon dioxide formed in the oxidation and absorbed in the receiver, special precaution had to be taken in the titration. The solution was titrated to the end-point, an excess of acid then added, the titration solution brought to the boiling point, and then titrated back to neutrality. This procedure was repeated until consistent results for the net amount of alkali used were obtained.

A control experiment was performed with the same solvents, reagents, and procedure. In order to arrive at the correct estimate of the volatile acids formed from carotene alone, the original value was corrected for the volatile acids formed in the control experiment. Special experiments demonstrated that the volatile acids in the controls were due to the small amounts of impurities in the reagents, especially in the potassium permanganate.

Experimental Data.

The results are summarized in Table I. From these results it is clear that there are 4 mols of acid formed by oxidation of carotene in the manner described.

TABLE I.

Volatile Acid from Oxidation of Carotene with Potassium Permanganate in Pyridine Solution.

	Carotene.		Xanthophyll.
Experiment No.	1	2	3
Aluminum used, *gm.*	3	5	5
Phosphoric acid used, *cc.*	0.5	1.0	1.0
Gm. of pigment	0.01300	0.01046	0.02005
Mols " "	0.0000242	0.00001949	0.00003527
Total titer, 0.01 N NaOH	12.87	10.01	17.34
Control, 0.01 N NaOH	3.10	2.17	3.80
0.01 N volatile acid, *cc.*	9.77	7.84	13.54
Mols acid: mols pigment	4.03	4.02	3.84

Since the completion of this work an article by Karrer and Helfenstein,[3] reporting this same value, has just appeared. As no experimental data are included in the article it is still uncertain what methods were employed by these workers.

Nature of the Acid.

(With Harold W. Milner.)

Dyer's method (3) was used to identify the volatile acid. This method was easily adapted to quantities one-tenth as large as

[3] Karrer, P., and Helfenstein, A., *Helv. Chim. Acta*, **12**, 1142 (1929).

those described in the original publication and was used by us in this micro form.

The solution from the titration of the total volatile acid was transferred, little at a time, to the distilling flask of the Dyer distillation apparatus and concentrated to 10 cc. 1 cc. of molar phosphoric acid was then added and the volume made up to the 15 cc. mark on the flask. The volatile acid was then distilled with steam from the flask, condensed in a silver tube and collected

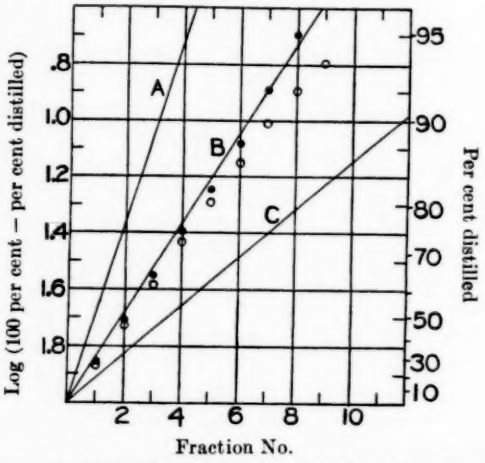


FIG. 1. Dyer's distillation curves for volatile acids. Comparison of values obtained with volatile acids from oxidation of carotene and xanthophyll. Curve A, propionic acid; Curve B, acetic acid; Curve C, formic acid. Acid from carotene oxidation ●; acid from xanthophyll oxidation ○. Distillate collected in 10 cc. fractions.

in 10.0 cc. portions. Each fraction was titrated with 0.01 N sodium hydroxide. The distillation of the volatile acids from the control experiment was carried out in the same manner. Thus a blank determination was obtained for each fraction.

The identification of the acid was made by comparing the per cent of the acid distilled in any given volume with the values obtained by Dyer.

The results are plotted in Fig. 1. The lines represent Dyer's values and the points show our results. From this it is obvious

that excepting carbonic acid, acetic acid is the only volatile acid formed by the oxidation of carotene. This chart was actually made by plotting the value log (100 per cent minus the per cent distilled) as ordinate against the volume distilled as abscissa. For convenience the per cent distilled is also given on the ordinate.

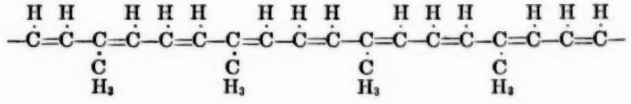
Acetic Acid from Xanthophyll.

Because of the great similarity shown by xanthophyll to carotene on hydrogenation (4), addition of iodine chloride (5) and of bromine (6), and by its reaction with benzoyl hydroperoxide (5) it seemed of importance to compare xanthophyll with carotene as to the number and kind of volatile acid molecules formed on oxidation.

The estimation was carried out in the manner described for carotene and it was found that approximately 4 mols of volatile acid are formed for each mol of xanthophyll oxidized. The results are tabulated in Table I, Column 3. Reference to Fig. 1 will show that the points determined by the micro modification of Dyer's method, lie very close to Dyer's curve for acetic acid. Therefore we conclude that aside from carbonic acid the only volatile acid formed from xanthophyll is acetic acid.

DISCUSSION.

Carotene and xanthophyll, on oxidation by this method, yield the same number of mols of acetic acid. This observation gives further support to the view that these two pigments are chemically closely related. Furthermore, the formation of 4 mols of acetic acid on oxidation clearly associates carotene with bixin for which a definite chemical structure has been put forward (7). On the basis therefore, of the similarity of carotene to other members of this group with respect to color (8), color reactions (9), and to the change of color on hydrogenation (10), and also on the theory that these compounds are built by condensation of isoprene nuclei which are subsequently dehydrogenated (11), it seems highly probable that a portion of the carotene molecule may have the following structure.



SUMMARY.

1. A method is described for determining the volatile acids formed by the oxidation of carotene and xanthophyll.

2. Each of these pigments has been shown to form 4 mols of acetic acid per mol of pigment on oxidation.

3. A structure for the conjugated system of double bonds in these pigments is proposed.

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AN APPARATUS FOR THE EVAPORATION OF LIQUIDS IN A TEST-TUBE.

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(Received for publication, February 4, 1930.)

The following procedure and apparatus were designed primarily to permit the evaporation of foamy liquids such as urine in 200 × 20 mm. test-tubes so that ashing and subsequent precipitation of one of the constituents of the solution could be carried out in the same tube.

The procedure consists in heating the test-tube containing the liquid to be evaporated in a suitable bath maintained approximately at the boiling point of the liquid to be evaporated while a gentle current of heated air is directed into the test-tube.

Evaporation of fluids that foam and bump badly by the ordinary procedure of boiling requires a large vessel such as a 250 cc. Kjeldahl flask, and when it is desired to transfer the liquid to a small vessel such as a 200 × 20 mm. test-tube at a volume of 10 cc. or less, the procedure is at least an awkward one and not likely to be quantitative.

By the means to be described, not only is it possible to precipitate in the original tube used for evaporation and ashing, but the use of material to prevent bumping such as silica or pebbles which might introduce uncertainty into the analysis is eliminated, because the evaporating liquid does not boil. Still another advantage results from the fact that once the tube is adjusted in place, almost no further attention is required until evaporation is complete.

Compressed air is passed through a cylinder 30 cm. high packed with absorbent cotton to free it from particles of dust. This is connected to an air heater consisting of a heating coil enclosed

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in a glass tube (Figs. 1 and 2). The glass tube is 4 cm. inside diameter by 20 cm. long and is fitted with a glass wool filter. The heating coil consists of 21 feet of 22 gauge nichrome resistance wire

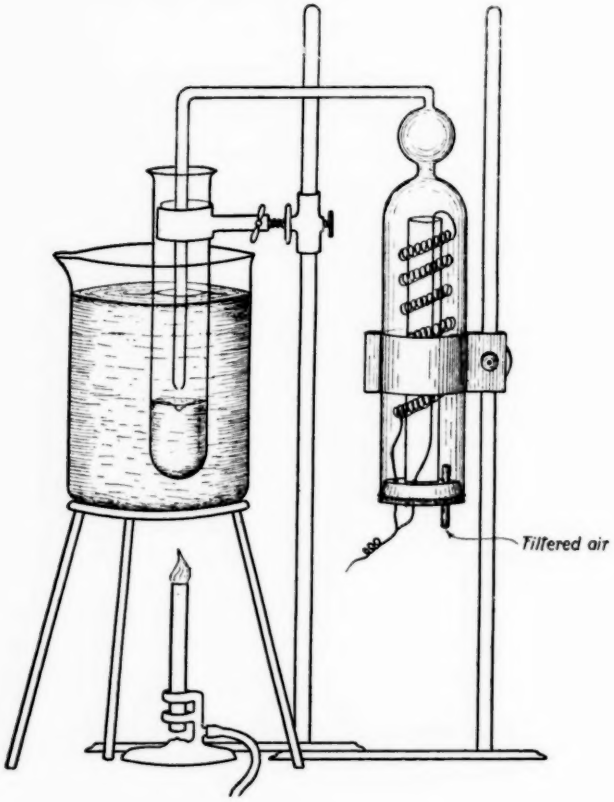

FIG. 1. Evaporation apparatus.

wound in a spiral. This is an ordinary laboratory heating unit for use on a 110 volt current. A cork stopper plugs the lower end of the heating apparatus. On this is mounted a piece of Pyrex tubing, 1.5 cm. in diameter and 16 cm. long, which holds the

heating coil. One section of the heating coil is straightened to pass down the interior of the Pyrex tubing on which it is mounted, and connections through the cork are made with short pieces of copper wire and binding posts. A layer of glass wool covers the cork. This keeps the lower end of the heater cool regardless of how long it is in operation. No rheostat is required. This is avoided by the simple expedient of attaching the heating unit of two heaters in series. The air temperature thus produce

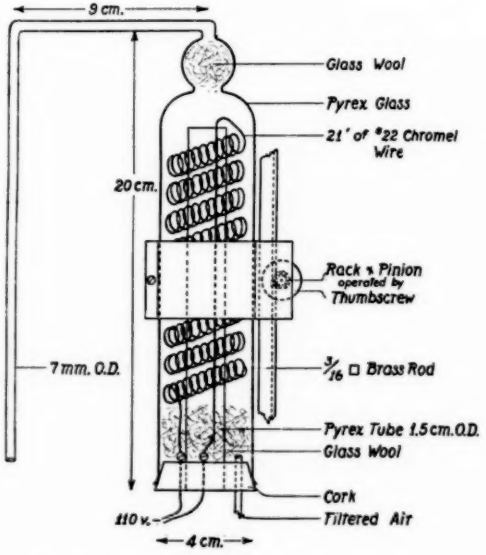

FIG. 2. Heating unit of evaporation apparatus.

(200–300°) is sufficient for the purpose. The heating unit is conveniently raised or lowered by a rack and pinion arrangement, shown in Fig. 2. More than 100 solutions have been evaporated with two such heaters during the last 2 years and they are still giving satisfactory service.

Usually the test-tube containing the liquid to be evaporated is heated in a bath of dilute sulfuric acid (boiling point 110–120°) when 30 or more cc. of urine are to be evaporated in each tube.

With dilute solutions, or when only 10 to 20 cc. of fluid are to be evaporated at a time, an ordinary water bath at 100° does very well. Fastest evaporation occurs when the outlet of the hot air tube is about 3 cm. above the surface of the liquid to be evaporated. If the outlet for the air is adjusted about once every 10 minutes, the rate of evaporation of urine is only slightly slower than is obtained by boiling in a Kjeldahl flask. 30 cc. can usually be evaporated in 35 to 40 minutes. Even if no attention is given the tube after it is once adjusted, 30 cc. of urine will evaporate in approximately an hour. The current of air required is only sufficient to produce a cup-like depression in the surface of the liquid.

A simple apparatus suitable when only a few determinations are required can be made from laboratory materials. The air from the absorbent cotton filter is directed by means of glass tubing against the interior lower portion of a Kjeldahl flask which is fitted with a *cork* stopper. A second glass tube, similar to the outlet of the electrical heater, conducts the air from the Kjeldahl flask to the surface of the liquid to be evaporated. A Bunsen burner under the flask heats the air passing through. The connection between the cotton filter and the flask should be made in part with rubber tubing so that the flask will not be rigid. If it is supported somewhat loosely in a clamp on a ring stand, the distance of the outlet from the surface of the liquid can be adjusted by sliding the entire flask down as the liquid evaporates.

The objection might be raised that dried material on the sides of the test-tube in which liquids are evaporated by this process might be carried out of the tube by the current of air. This objection cannot be applied in the case of urine because it dries to a gummy mass which sticks to the sides of the glass, as do many other biological fluids. It is not likely that the gentle current of air used would cause a loss except from material that dried to a fine powder. The method has been tried out with urine, trichloroacetic acid blood filtrate, etc., in connection with the determination of calcium and magnesium. Recovery, as indicated by control analyses in which the evaporation apparatus was not used, has always been complete.

AGE AND THE EFFECT OF UNUSUAL DIETS.*

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(Received for publication, January 25, 1930.)

Experiments have been reported by Cox, Smythe, and Fishback (1) in which they found that diets containing from 0.3 to 0.9 per cent of cystine produced severe renal degeneration in young rats of 50 gm. body weight or less while half grown rats showed no deleterious effects from such diets. The inference is made that cystine is more toxic for young than for old rats. We believe that this apparent difference may be explained in another manner. Data upon this point and upon the age effect of other unusual diets are reported here.

Addis, MacKay, and MacKay (2) found that an otherwise normal diet containing 1.0 per cent of cystine could be administered to albino rats from 30 to 360 days of age without any discernible ill effect. In Fig. 1 the food intake[1] for one of these groups of rats has been plotted against age. As might be expected the food intake per day decreases with increasing age. The cystine intake which is also charted must likewise decrease as the rats grow older. These rats weighed 55 to 60 gm. when the cystine diet was commenced. The young rats used by Cox *et al.* weighed

* This investigation was made possible by the Edward N. Gibbs' Prize Fund of the New York Academy of Medicine.

[1] Body surface is determined from the body weight, corrected for the intestinal contents, by using Meeh's formula with the constant of Carman and Mitchell (3). Although the results would be essentially the same for the present purpose, body surface has been chosen rather than body weight as a standard for comparison of the daily food intakes because it is a more reasonable measure of body size when considering the energy requirement and is particularly suitable in the present instance because the amount of renal tissue is directly proportional to the body surface (4).

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considerably less than this and were therefore presumably younger. It is reasonable to suppose that the food intake of these rats, in relation to their size, may have been considerably higher than that of our 60 gm. rats when they were first given the cystine diet. Even with only 0.6 per cent instead of 1.0 per cent their cystine intake may consequently have been significantly higher. In order to test this point an experiment was carried out in which diets containing different amounts of cystine were fed to small groups of rats at various ages. The control diet with which the

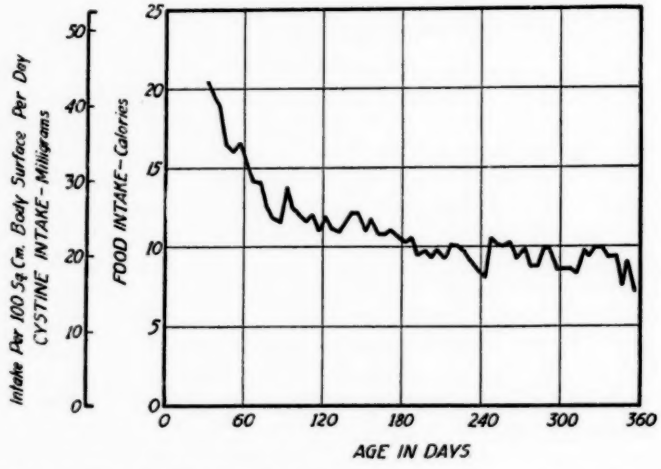

FIG. 1. Mean food intake of a group of six male rats on an adequate diet with a value of 4.8 calories per gm. and containing 1 per cent cystine.

cystine was mixed has been described elsewhere (2). The gross appearance of the kidneys when the animals were killed was used as an indicator of toxic effect from the cystine. Only when the organs were gray or white and swollen was a toxic effect noted. A check upon these observations was had in the blood urea concentration. Whenever a positive result is recorded the blood urea concentration was found to have increased. The results of the experiments are detailed in Table I.

These results do not show that cystine is any more toxic for

young than for older rats. With a diet containing a given concentration of cystine the young rats, since they require more calories for their size, eat a greater quantity of food and it follows that their cystine intake is greater than that of the adults. They may thus receive a toxic dose of cystine each day while older rats on the same diet, because they eat less for their size, receive cystine in

TABLE I.

Toxic Effect of Cystine Diets for Male Rats.

No. of rats.	Mean body weight.	Days of age when on diet.	Average food intake per 100 sq. cm. body surface per day.	Cystine content of diet.	Average cystine intake per 100 sq. cm. body surface per day.	Evidence of toxic effect.*
	gm.		*gm.*	*per cent*	*mg.*	
3	48	22–28	6.89	0.1	7	0
4	46	22–28	7.41	0.6	44	+
4	41	22–28	6.03	1.0	60	+
3	38	22–26	5.12	2.0	102	+
3	183	60–70	3.22	1.0	32	0
3	169	60–70	3.38	2.0	68	+
3	151	60–67	2.84	3.0	85	+
3	271	130–140	2.43	1.0	24	0
4	273	130–140	2.38	2.0	48	+
3	262	130–138	2.19	3.0	66	+
3	351	225–235	2.10	1.0	21	0
3	328	225–235	1.70	2.0	34	0
3	334	225–235	1.59	3.0	48	+

* The result in this column is judged from the majority of the group. Except for two or three cases the result was in one direction for the whole group. In the groups in which a negative effect is recorded the kidneys were normal in all members of the group.

harmless doses. This explanation of the results of Cox, Smythe, and Fishback also explains their observation that some rats on their cystine diet eventually recovered and grew normally without any change in the ration. When the rats first show toxic effects their food consumption and hence cystine intake decreases and at the same time their usual food intake is decreasing each day as they grow older. If they do not die before the point is reached

where their food intake has decreased so that a non-toxic cystine intake follows they may then be expected to grow normally just as do older rats on a 1.0 per cent cystine diet. If there is an age difference in the toxicity of fed cystine it must be of very small magnitude and is not demonstrable with the relatively gross methods which have been used here or by Cox, Smythe, and Fishback (1).

In a preliminary report (5), which was made several years ago, of some experiments dealing with the influence of age on the relation between the diet protein and the size of the kidneys, we were led astray in a manner very similar to that of Cox, Smythe, and

TABLE II.

*Protein Intake in Gm. per 100 Sq. Cm. of Body Surface per Day.**

Concentration of protein in diet.	Age in days.			
	70	130	220	400
per cent				
12	0.45			0.19
18	0.61	0.43	0.37	0.38
25	0.77			
31	0.95			0.56
43	1.30	1.03	0.94	
55	1.72			
67	2.49	1.83	1.79	1.34

* The figures given here are averages obtained on groups of thirteen to twenty-nine rats.

Fishback and for a like reason. From the bare results presented at that time we were inclined to believe that an increase in protein consumption has less effect upon the weight of the kidneys in old than in young rats. Further analysis (6, 7) of our data has shown that this is not the case and that age is without demonstrable effect upon the relation between the protein intake and the weight of the kidneys. The apparent effect which we at first believed to exist resulted, just as in the case of cystine results which have been discussed, from failure to take cognizance of the very different caloric and hence protein intakes in relation to body size at different ages. This is shown very well in the results given in Table II for male rats of various ages. Just as a higher

cystine content is required to give an old rat the same intake as a young rat, an 18 per cent protein content in the diet gives a 70 day old rat the same protein intake as a 400 day old rat obtains from a 31 per cent protein diet and so on.

Many other examples such as have been presented here, of the apparent different effect of unusual diets at various ages, have been observed during the course of an extended study of the effect of diet on the kidney. When considered in the light of the present experiments they demonstrate how important it is to exercise great care in attributing directly to difference in age the different results obtained in feeding experiments at various ages. That age differences do exist is very probable but the indirect effect of the variation in the food consumption: body size ratio which is associated with age must be ruled out before the result may be considered dependent upon a more fundamental age difference. The necessity for determining the food intake in all types of feeding experiments, a point often emphasized by Mitchell (8) is evident. Another obvious suggestion which arises from the present study is the much greater ease with which the effects of unusual diets may be observed in young animals because of the relatively low concentration which need be included in their diet to give them a significant intake of the added ingredient. This is true of course only when the effect of an added ingredient is under observation. When an inadequate diet is being considered just the reverse may be the case. At a given concentration of a necessary food substance in a diet, young rats, due to their higher food intake, might easily receive a sufficient amount while older rats, conceivably needing a similar quantity of this ingredient for their size but taking relatively fewer calories, have an insufficient intake. In other words a diet which may be adequate for a young rat is not necessarily adequate for the adult. It is possible that the different results obtained in regard to kidney damage from high protein diets may be an example of this type. The majority of investigators (2) agree that high protein diets give no evidence of renal damage. Two observers (9, 10) who report pathological changes in the kidney following long continued feeding of high protein diets note that they do not occur in young but only in old rats. A diet containing a high concentration of

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protein leaves little place for the mineral content and vitamin-containing foods and there is some evidence (11) that one of the latter should bear a constant relation to the protein intake. It is quite possible that some high protein diets may furnish sufficient essential non-caloric ingredients at the level of the high food intake of young rats and yet be inadequate for old rats where the food intake is much lower. A concrete example is presented in Fig. 2. It happens that in this case the diet must have been

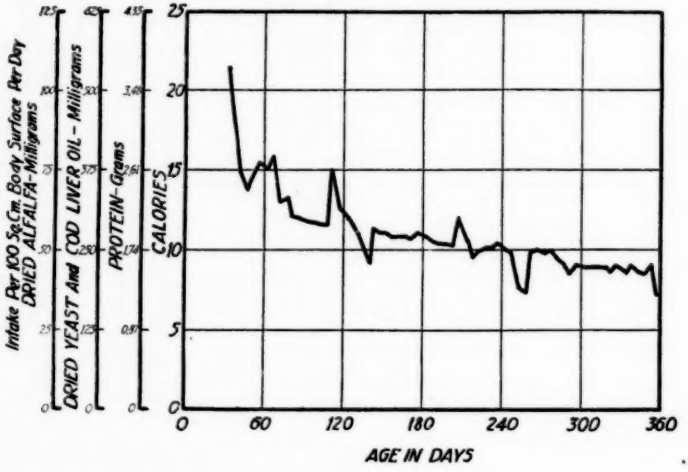

FIG. 2. Mean food intake of a group of six male rats on a high protein diet containing 69.5 per cent protein, 10 per cent cod liver oil, 10 per cent dried yeast, 2 per cent alfalfa, and 4.0 per cent of the Osborne and Mendel salt mixture (12) based upon the mineral content of milk.

adequate at all ages for no renal damage resulted. In view of the divergence of opinion as to the ability of high protein diets to produce renal damage it would seem that in the future this point should be considered so as to determine whether or not the renal damage which has been reported as due to high protein diets is a result of the high protein intake *per se* or is due to the relative inadequacy of the diet at certain ages.

SUMMARY.

Evidence is presented which indicates that cystine added to the diet is no more toxic for young than for old rats. The apparent difference in the effect appears to be due to the larger dose received by the young rat because its food and hence cystine intake is higher in relation to body size than that of the adult. Likewise a high protein diet seems to produce a greater change in the kidney weight of young than of old rats because the food and therefore protein intake of the former is much greater. These results are considered in relation to the effect of unusual diets and age in general.

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